IN SILICO DOCKING IN VITRO PROTECTIVE EFFECT OF CERTAIN SYNTHESIZED FLAVONOIDS IN DOXORUBICIN_INDUCED MYOCARDIAL INFARCTION IN RATS

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INTRODUCTION

Myocardial infarction

Myocardial infarction (MI, heart attack, coronary thrombosis) occurs when a coronary vessel becomes occluded for more than 6 hours[1], it is the irreversible necrosis of heart muscles secondary to prolonged ischemia, usually results from an imbalance of oxygen supply and demand[2].

It is one of the serious disorders among ischemic heart diseases invariably followed by several biochemical alterations such as hyperlipidemia, lipid peroxidation, free radical damage, thrombosis, etc., leading to qualitative and quantitative alteration of myocardium. The appearance of cardiac enzymes in the circulation generally indicates myocardial necrosis [3].

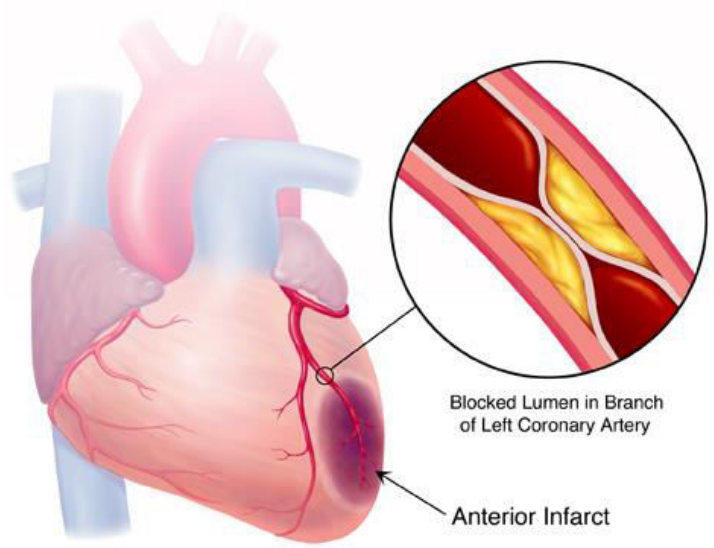


Fig 1: Myocardial infarction in left coronary artery

Prevalence rate of myocardial infarction

Globally, cardiovascular diseases constitute a leading cause of mortality. The Incidence and prevalence of myocardial infarction progressively increased with age during the later half of the last century[2]. Developing countries like India are also struggling to manage the impact of cardiovascular diseases (CVD) along with the growing burden of obesity[4]. It may account for one third of the deaths by the year 2020. Current projections suggest that India will have the largest CVD burden in the world by the year 2020[5].

Clinical classification of myocardial infarction

For the sake of immediate treatment strategies, such as reperfusion therapy, it is usual practice to designate MI in patients with chest discomfort, or other ischaemic symptoms that develop ST elevation in two contiguous leads, as an 'ST elevation MI' (STEMI). In contrast, patients without ST elevation at presentation are usually designated as having a 'non-ST elevation MI'(NSTEMI). Many patients with MI develop Q waves (Q wave MI), but others do not (non-Q MI). Patients without elevated biomarker values can be diagnosed as having unstable angina. In addition to these categories, MI is classified into various types, based on pathological, clinical and prognostic differences, along with different treatment strategies[6].

Spontaneous myocardial infarction (M1type 1)

This is an event related to atherosclerotic plaque rupture, ulceration, fissuring, erosion, or dissection with resulting intraluminal thrombus in one or more of the coronary arteries, leading to decreased myocardial blood flow or distal platelet emboli with ensuing myocyte necrosis. The patient may have underlying severe CAD but, on occasion (5 to 20%), non-obstructive or no CAD may be found at angiography, particularly in women[6].

Myocardial infarction secondary to an ischaemic imbalance (M1 type 2)

In instances of myocardial injury with necrosis, where a condition other than CAD contributes to an imbalance between myocardial oxygen supply and/or demand, the term 'MI type 2' is employed (Figure 2). In critically ill patients, or in patients undergoing major (non-cardiac) surgery, elevated values of cardiac biomarkers may appear, due to the direct toxic effects of endogenous or exogenous high circulating catecholamine levels. Also, coronary vasospasm and/or endothelial dysfunction have the potential to cause MI[7].

Cardiac death due to myocardial infarction (M1 type 3)

Patients who suffer cardiac death, with symptoms suggestive of myocardial ischaemia accompanied by presumed new ischaemic ECG changes or new LBBB but without available biomarker values represent a challenging diagnostic group. These individuals may die before blood samples for biomarkers can be obtained, or before elevated cardiac biomarkers can be identified. If patients present with clinical features

of myocardial ischaemia, or with presumed new ischaemic ECG changes, they should be classified as having had a fatal MI, even if cardiac biomarker evidence of MI is lacking[7].

Myocardial infarction associated with revascularization procedures (MI types 4 and 5)

Periprocedural myocardial injury or infarction may occur at some stages in the instrumentation of the heart that is required during mechanical revascularization procedures, either by PCI or by coronary artery bypass grafting (CABG). Elevated cTn values may be detected following these procedures, since various insults may occur that can lead to myocardial injury with necrosis. It is likely that limitation of such injury is beneficial to the patient: however, a threshold for a worsening prognosis, related to an asymptomatic increase of cardiac biomarker values in the absence of procedural complications, is not well defined. Subcategories of PCI-related MI are connected to stent thrombosis and restenosis that may happen after the primary procedure[8].

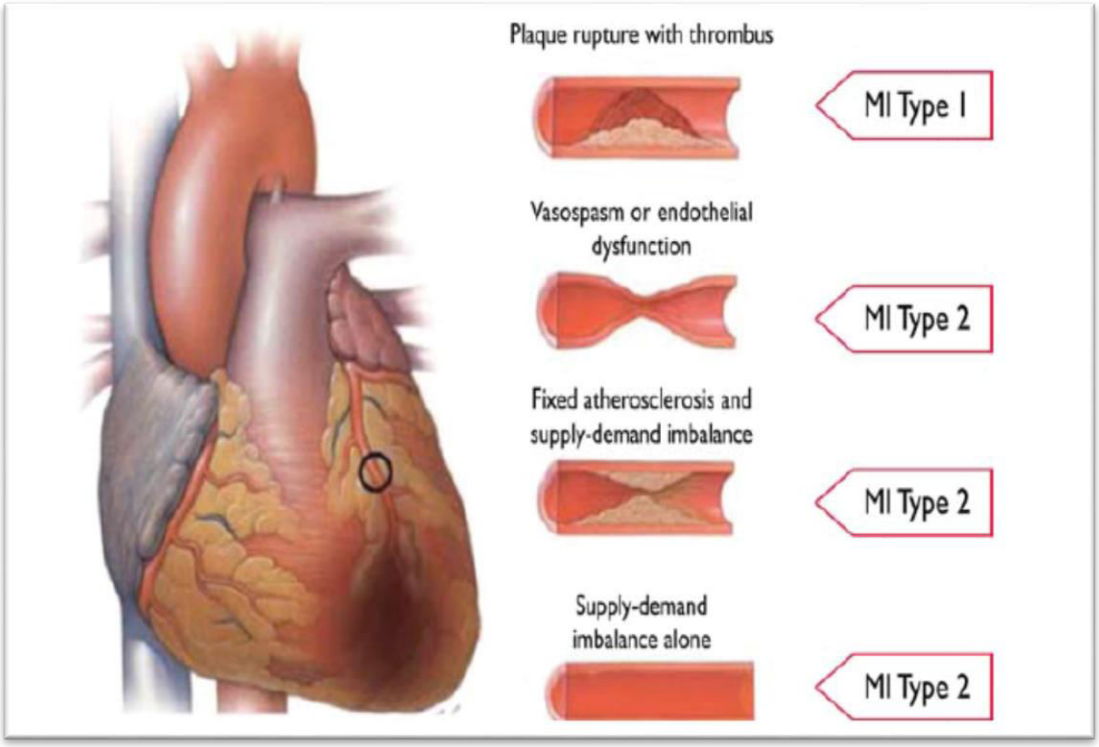


Fig 2: Clinical classification of myocardial infarction

Pathophysiology of myocardial infarction

Initiating events in MI

Post-mortem examinations after MI almost invariably show advanced coronary atherosclerosis with a thrombotic occlusion in one vessel. 'Sudden ischaemic death' within an hour or so of the onset of symptoms, before infarction proper can develop, also occurs. This is probably due to ventricular fibrillation. However, these patients usually also have obstructive lesions[1].

Stress-induced acute abnormalities in both clotting factors and platelets have been proposed, but it is currently thought that a particularly lipid-rich plaque, with low amounts of smooth muscle and fibrous support, may fissure or rupture. This exposes lipid and sub-endothelial structures, triggering massive platelet aggregation and subsequent thrombosis. In the few cases where no substantial atheroma is found on angiography or at post-mortem examination, the cause may be severe vasospasm or a primary platelet or clotting abnormality[1].

Severity of MI

Ischaemia is a deficiency of blood supply to tissues, if the deficiency is sufficiently severe and prolonged, the tissue eventually necrosis. The most common general cause is a failure of blood flow resulting from obstruction or cardiovascular insufficiency[1].

When arteries are chronically inflamed (arteritis), the artery wall may be permanently damaged by the neutrophil infiltration and necrosis. If this involves small arteries, the entire arterial wall is affected and complete occlusion of the lumen may occur. If a larger artery is affected, only part of the wall may be damaged and blood is still able to pass. Healing subsequently occurs with the formation of scar tissue, which may weaken the artery wall and produce an aneurysm (bulge) that may eventually rupture. A common cause of vascular obstruction is atherosclerosis, which affects the intimal lining of the artery wall, particularly in medium to large arteries. Atheromatous plaques are laid down that partially occlude the lumen and become sites for thrombus formation. In contrast, arteriosclerosis affects the media of the arterial wall, which becomes hard and inelastic and small arterioles may become occluded[1].

A thrombus (blood clot) may be formed over the site of an atheromatous plaque in an artery. Thrombi may also form in large veins, usually in the region of valves, owing to stasis of blood. If a venous thrombus in the leg (deep-vein thrombosis), or a

fragment of it, breaks away from its site of formation, it will travel downstream through veins of increasing diameter, through the heart and into the pulmonary tree, until it lodges in a small artery. This obstruction to the circulation is known as an embolus, i.e. a clot or clot fragment derived from a blood clot formed at one site, which lodges in another. Because it is often impossible to distinguish between an embolus and a thrombus, and because it does not affect treatment, it is usual to speak of thromboembolic disease[1].

The site of formation of the original clot determines the organ eventually affected, which may be predicted on the basis of the anatomy of the vascular tree. Emboli can also be due to air introduced into the bloodstream in advertently during IV therapy (air embolus) or may be the result of deep diving, causing nitrogen emboli if the diver rises to the surface too rapidly, causing the divers' syndrome known as the bends. Fat droplets released from the site of a fracture (fat embolus) do not cause an infarction as such, but can result in a severe interruption of gas exchange if deposited in the lung. Thrombosis in a coronary artery may itself cause a myocardial infarction or may throw off an embolus that travels further into the coronary arterial tree to obstruct a smaller vessel and so affect a smaller area of heart muscle. Emboli formed on damaged heart valves can reach the retina, affecting sight, whereas those resulting from atrial fibrillation tend to cause strokes by occluding a cerebral artery[1].

Small thrombo emboli are quite quickly dissolved by natural clot-dissolving factors derived from blood plasminogen (plasmin), red cells and vessel walls, e.g. tissue-type plasminogen activator. Temporary interruptions of CNS function, known as transient ischaemic attacks (TIAs), are common and usually last less than 15 min, but may persist for up to 24 h. Circulatory brain obstructions of longer duration are classed as strokes[1].

Acute MI is treated in the early stage with fibrinolytic (thrombolytic) drugs, e.g. *alteplase*, *reteplase*, *tenectoplase* and *streptokinase*, and the latter is also used in several other thromboembolic situations. All of these are unsuitable for use in early stroke unless it is certain that the stroke has not been caused by a cerebral haemorrhage, which would be exacerbated by clot dissolution. Constriction of the vascular smooth muscle(vasospasm) may occur in coronary arteries, as in variant angina and in peripheral arteries, causing Raynaud's disease. Poor perfusion of tissue may also arise from circulatory insufficiency. If cardiac output is low because of heart failure or

arrhythmia, the blood supply to many tissues will be reduced. This may also occur if the blood volume is low, perhaps following severe blood loss, causing shock.

If a tissue undergoes a period of anoxia, then irreversible damage occurs, followed by wound healing and organization of scar tissue. Scar tissue can never fulfil the functions of the tissue it replaces. In the heart, this means that as well as being noncontractile, the infarcted area is inelastic and poorly conducting. This has the following potential consequences:

- Poor contractility leads to poor ejection, i.e. systolic failure.
- Poor elasticity (reduced compliance) leads to poor filling, i.e. diastolic failure.
- Poor conductivity leads to arrhythmias.

The consequences in individual cases depend primarily on the size of the area of myocardium served by the coronary vessel that is occluded. The mildest form involves a small arteriole, resulting in a clinically silent (symptomless) infarction. Moreover, dilatation of neighbouring vessels by autoregulation may protect there an adjacent to the ischaemic core from complete anoxia, thereby limiting infarct size. However, if this is repeated over a long period it results in widespread 'patchy fibrosis' and eventual cardiac failure. Occlusion of larger arterioles will cause a classical presentation of MI, but if there a damaged is not too extensive the patient will survive, possibly with a degree of permanent cardiac failure. At its most severe an MI may involve one of the main coronary arteries, often the left anterior descending, which supplies most of the left ventricle, causing an anterior infarct. Death is likely if more than about 50% of the left ventricle is damaged[1].

One important factor determining outcome is how well developed the patient's collateral coronary vessels are; another is how much conducting tissue is involved. Conduction across the whole myocardium is necessary for normal coordinated contraction, and ischaemic muscle may conduct erratically. In addition, ischaemic damage to nodal tissue or nerve tracts may have a disproportionate effect because arrhythmias can compromise the function of the entire heart[1].

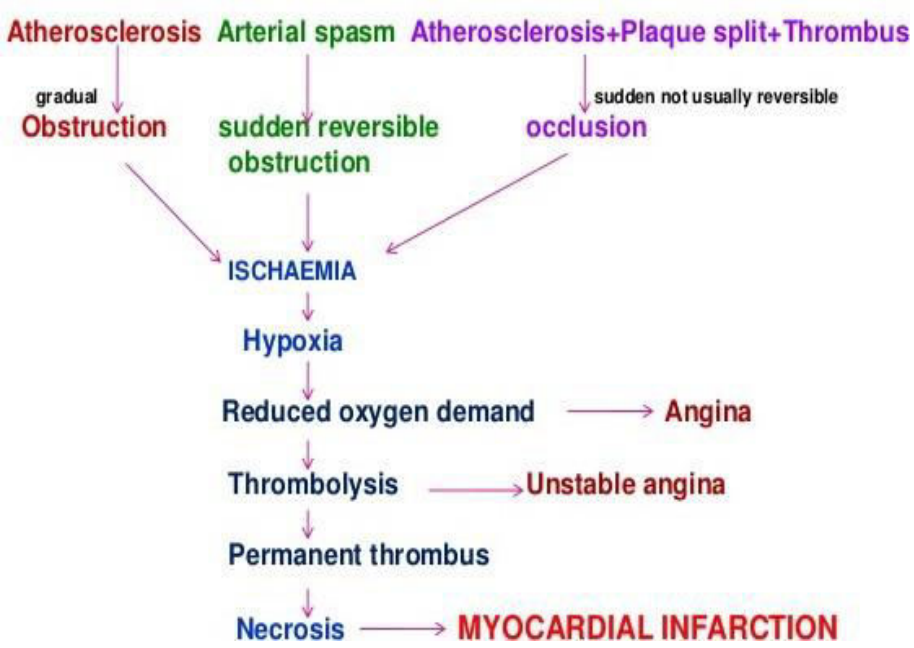


Fig 3: Pathophysiology of MI

Etiology of myocardial infarction

Myocardial infarction (MI) results from lack of oxygen supply to the working myocardium. Regional infarcts are due to lack of blood flow that occurs when an epicardial artery is blocked by atheroma or thrombus, or other obstructions. Global subendocardial infarcts occur when there is lack of oxygenation despite circulation for example, when there is a respiratory arrest followed by prolonged hypoxemia[9].

Cardiac markers in the diagnosis of myocardial infarction

The analysis of cardiac biomarkers has become the frontline diagnostic tools for AMI, and has greatly enabled the clinicians in the rapid diagnosis and prompt treatment planning, thereby reducing the mortality rate to a great extent[10].

- Creatine kinase
- Lactate dehydrogenase (LDH)
- Troponins
- Myoglobin

Creatine kinase

It is an enzyme chiefly found in the brain, skeletal muscles, and heart. An elevated level of creatine kinase is seen in heart attacks, when the heart muscle is

damaged, or in conditions that produce damage to the skeletal muscles or brain. There are three different forms of creatine kinase that can be measured[10].

- CK-MM (located in the skeletal muscles and heart)
- CK-MB (mainly located in the heart)
- CK-BB (located in the brain)

An elevated level of creatine kinase, specifically CK-MB, occurs within hours of a heart attack as the heart muscle cells die. The enzyme level continues to rise for the first 18 to 24 hours after a heart attack and slowly returns to normal after a few days[10].

Trauma and other conditions that damage the skeletal muscle are also associated with an elevated creatine kinase level. In some cases, the test may be used to detect muscle conditions such as polymyositis (condition characterized by the inflammation of muscles) or to estimate the degree of muscle damage. Stroke and other forms of brain damage can also result in an elevated creatine kinase level[10].

Creatine kinase (CK) has several functions in cellular energy metabolism. It catalyzes the reversible transfer of high-energy phosphate from ATP to creatine, facilitating storage of energy in the form of phosphocreatine. In muscle cells, this extra energy buffer plays a pivotal role in maintaining ATP homeostasis[10].

Lactate dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme present in many different cells. There are five isoenzymes, each with different specificities for different types of tissue. In the case of cardiac injury, LDH isoenzyme1 is higher than isoenzyme-II. But under normal circumstances, isoenzyme II is present in greater amounts than isoenzyme I. The LDH starts going up in 12-24 hours following an MI, and it dissipates within a week or two. This test has been supplanted by the other markers[10].

Troponins

Troponins are the best overall markers; they have the best combination of sensitivity, specificity, and ease of test performance of all the markers. They are

complex of 3 protein subunits located on the thin filaments of the skeletal and cardiac muscle fibers. They are;

- Troponin C -Calcium-binding component in the skeletal and cardiac muscle, but it is not extremely specific for myocardial injury.
- Troponin T - Tropomyosin-binding component
- Troponin I - Inhibitory component

The isoforms of troponin-T and troponin-I differ in the skeletal and the cardiac muscle, and thus are extremely specific for cardiac tissue necrosis. Troponin-T is present chiefly in the bound form to the contractile elements of the myocardial cells and it is also present free in the cytoplasm[10].

Troponin-T exhibits a dual release initially of the cytoplasmic component and later of the bound component. Troponin-I is extremely specific for the cardiac muscle and has not been isolated from the skeletal muscle. This absolute specificity makes it an ideal marker of myocardial injury. They are released into the circulation 6-8h after myocardial injury, peak at 12-24h and remain elevated for 7-10days[10].

Troponins are components of cardiac muscle that are released into the blood when myocardial cells are injured. They are very specific for myocardial muscle – even more specific than CK-MB. Troponins go up within 3-12 hours after the onset of MI[10].

Myoglobin (MYO)

Myoglobin is a small cytoplasmic oxygen-binding protein found in the skeletal as well as the cardiac muscle. It is released extremely early into the serum, 1h after the onset of myocardial injury, peaks at 4-12h and returns to baseline values immediately[10].

The major disadvantage of myoglobin is the lack of specificity to the cardiac tissue due to the presence of large amounts of MYO in the skeletal muscle. The levels of MYO can therefore not be used as a single diagnostic marker, but in conjunction with the troponins or CK-MB. Thus, serum levels of MYO can be used to rule out, rather than diagnose, myocardial infarction[10].

Role of liver enzymes related to myocardial infarction

ALT means alanine transaminase, it is a liver enzyme. ALT helps to metabolize protein. This liver enzyme become release into bloodstream when liver becomes damage. An increase level of ALT into blood may indicate hepatic disorder, liver tumor, infection or toxic hepatitis. Acute hemolytic anemia, myocardial infarction also may occur due to elevated level of liver enzyme into blood[10].

ALP means alkaline phosphatase, it is another liver enzyme. This liver enzyme needed little amount for trigger specific chemical reaction. ALP normally present in liver, bone, kidney and intestine. Elevated level of ALP may indicate gallstone disease, drug induce hepatitis or biliary tumors[10].

AST means Aspartate transaminase. This liver enzyme plays an important role in the metabolism of amino acid alanine. Elevated level of AST may indicate active cirrhosis disease, infection or toxic hepatitis or acute hemolytic anemia. Crushing injuries, heart attack and tumor can occur due to increase of liver enzyme into blood[10].

Management of myocardial infarction

- Act promptly to save life and reduce complications
- Treat acute symptoms
- Restore flow through the affected artery (revascularization)
- Minimize subsequent infarct size
- Treat complications
- Rehabilitate
- Ensure secondary prevention of subsequent

Initial therapy for acute myocardial infarction is directed toward restoration of perfusion as soon as possible to salvage as much of the jeopardized myocardium as possible. This may be accomplished through medical or mechanical means, such as percutaneous coronary intervention or coronary artery bypass grafting[11].

Further treatment is based on;

- Restoration of the balance between the oxygen supply and demand to prevent furtherr ischemia

- Pain relief
- Prevention and treatment of any complications that may arise.

Thrombolytic therapy has been shown to improve survival rates in patients with acute myocardial infarction if administered in a timely fashion in the appropriate group of patients. If percutaneous coronary intervention (PCI) capability is not available or will cause a delay greater than 90 minutes, then the optimal approach is to administer thrombolytics within 12 hours of onset of symptoms in patients with ST-segment elevation greater than 0.1 mV in 2 or more contiguous ECG leads, new left bundle-branch block (LBBB), or anterior ST depression consistent with posterior infarction. Tissue plasminogen activator is superior to streptokinase in achieving a higher rate of coronary artery patency; however, the key to efficacy lies in the speed of the delivery of therapy[11].

Aspirin and antiplatelet therapy

Aspirin has been shown to decrease mortality and re-infarction rates after myocardial infarction. Administer aspirin immediately, which the patient should chew if possible upon presentation. Continue aspirin indefinitely unless an obvious contraindication, such as a bleeding tendency or an allergy, is present. Clopidogrel may be used as an alternative in cases of a resistance or allergy to aspirin. Clopidogrel as Adjunctive Reperfusion Therapy Thrombolysis in Myocardial Infarction suggest that adding clopidogrel to this regimen is safe and effective[11].

Heparin and other anticoagulant agents

Heparin has an established role as an adjunctive agent in MI patients, Heparin is also indicated in patients undergoing primary angioplasty. Few data exist with regard to efficacy in patients not receiving thrombolytic therapy in the setting of acute myocardial infarction. Bivalirudin (a direct thrombin inhibitor) has shown some promise in the setting of STEMI if combined with high-dose clopidogrel load and may be an appropriate alternative strategy[11].

Nitrates

Nitrates have no apparent impact on mortality rate in patients with ischemic syndromes. Their utility is in symptomatic relief and preload reduction. Administer to all patients with acute myocardial infarction within the first 48 hours of presentation, unless contraindicated (ie, in RV infarction)[11].

ACE inhibitors

They reduce mortality rates after myocardial infarction. Administer ACE inhibitors as soon as possible as long as the patient has no contraindications and remains in stable condition. They have the greatest benefit in patients with ventricular dysfunction. Continue ACE inhibitors indefinitely after myocardial infarction. Angiotensin-receptor blockers may be used as an alternative in patients who develop adverse effects, such as a persistent cough, although initial trials need to be confirmed[11].

Beta-blockers

They reduce the rates of reinfarction and recurrent ischemia. Administer to patients with myocardial infarction unless a contraindication is present. However, a large chinese trial showed no benefit to beta-blockade. This has created some doubt as to the benefit and may lead to a change in the guidelines[11].

Role of free-radicals and anti-oxidants in myocardial infarction

The role of free radicals in mediating myocardial tissue injury during myocardial ischemia and in particular during the phase of myocardial re-oxygenation. Associated with myocardial ischemia and reperfusion is the generation of oxygen-derived free radicals from a variety of sources that include the mitochondrial electron transport chain; the biosynthesis of prostaglandins; the enzyme xanthine oxidase; and circulating elements in the blood, with the polymorphonuclear neutrophil assuming a primary focus of attention[12].

Free radicals contain one or more of unpaired electrons. They play an important role in the pathogenesis of tissue damage in many clinical disorders. Oxygen free radicals are capable of damaging compounds of all biochemical classes; including nucleic acids, proteins, lipids, lipoproteins, carbohydrates and connective tissue

macromolecules (1). Normally, there is a balance between tissue oxidant and antioxidant activity. The latter is achieved by the antioxidant scavenger system, which includes enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and antioxidant vitamins (C, A, E and other carotenoids) (2). Oxidative stress is a condition in which oxidant metabolites exert their toxic effect because of an increased production or an altered cellular mechanism[13].

Experimental studies have shown that free radical scavengers (e.g., N-[2-mercaptopropionyl] glycine) and enzymes that scavenge or degrade reactive species of oxygen (superoxide dismutase or catalase) can reduce the mass of myocardial tissue that undergoes irreversible injury. Additionally, allopurinol, which inhibits the enzyme xanthine oxidase, reduces ultimate infarct size, putatively by reducing the xanthine oxidase generation of superoxide anion. Neutrophils that enter the ischemically injured myocardium under the influence of chemotactic attraction and activation of the complement system generate and release highly reactive and cytotoxic oxygen derivatives that are destructive to the vascular endothelium and to the cardiac myocytes[12].

Biological activity of flavonoids

They are group of polyphenolic compounds having benzo-***γ***-pyrone structure and are present in plants. They are synthesized by phenyl-propanoid pathway. They are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization[13].

Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals or by chelating metal ions. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules. As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems. Flavonoids have ability to induce human protective enzyme systems[13].

The number of studies has suggested protective effects of flavonoids against many infectious like bacterial and viral diseases, degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases[13].

Flavonoids also act as a secondary antioxidant defence system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centres of ROS generation. They also regulate growth factors in plants such as auxin. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids[13].

Chemistry of flavonoids

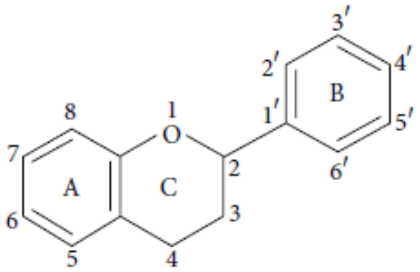


Fig 4: Basic nucleus of flavonoids

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from oranges. At that time it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on it became clear that this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified[13].

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B) linked via a heterocyclic pyran ring (C). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings[13].

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is aglycone. Six-member ring condensed with the benzene ring is either a α-pyrone (flavonols and flavanones) or its dihydroderivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and iso0-flavonoids (3-position). Flavonols differ from flavanones by hydroxyl group at the 3-position and a C2–C3 double bond[13].

Flavonoids are often hydroxylated in positions 3, 5, 7, 2, 3, 4, and 5. Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, gluco-rhamnose, galactose, or arabinose[13].

Importance of flavonoids in plants

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from oranges. At that time it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on it became clear that this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified. They are a major colouring component of flowering plants. Flavonoids are an integral part of human and animal diet. Flavonoids cannot be synthesized by humans and animals. Thus flavonoids found in animals are of plant origin rather than being biosynthesized in situ[13].

Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes. Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. They exhibit several pharmacological activities such as anti-allergic, anti-inflammatory, anti-microbial, anti-oxidant, cardioprotective, neuroprotective, hepatoprotective, anti-carcinogenic, anti-HIV property. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for the variety of pharmacological activities. They are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection[13].

The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds[13].

Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals or by chelating metal ions. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules. As a dietary component, flavonoids are thought to have health-promoting properties due

to their high antioxidant capacity both *in vivo* and *in vitro* systems. Flavonoids have ability to induce human protective enzyme systems[13].

The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases. The mechanisms involved in protection provided by flavonoids are described separately in this review. Flavonoids also act as a secondary antioxidant defence system in plant tissues exposed to different abiotic and biotic stresses[13].

Flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors in plants such as auxin. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids. This review deals with the structural aspects of flavonoids and their protective roles against many human diseases[13].

Classification of flavonoids

Over 5000 naturally occurring flavonoids have been characterized from various plants. Flavonoids can be divided in to variety of classes such as

- Flavone (flavone, apigenin, and luteolin)
- Flavonol (quercetin, kaempferol, myricetin, and fisetin)
- Flavanone (flavanone, hesperedin, and naringenin)
- Isoflavone
- Anthocyanidin

Pharmacological actions of flavonoids

- Antioxidant Activity
- Lipid peroxidation
- Hepatoprotective Activity
- Anti-Inflammatory Activity
- Anticancer Activity
- Antiviral Activity

Antioxidant Activity

Antioxidant Activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability. The B ring hydroxyl

configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and peroxy-nitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical. Mechanisms of antioxidant action can include suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; scavenging ROS; and upregulation or protection of antioxidant defences[13].

Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succin-oxidase, NADH oxidase[14].

Lipid peroxidation in MI

It is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanisms. Free metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (Fl-OH) are thermodynamically able to reduce highly oxidizing free radicals, such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation. Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at specific positions of different rings of flavonoid structures. The binding sites -catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trait of flavonoids makes them most effective scavengers of peroxyl, superoxide, and Peroxy-nitrite radicals[15].

Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation in vitro. Because of oxidation on the B ring of flavonoids having catechol group a fairly stable ortho- semiquinone radical is formed which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals exhibit weak scavenging potential. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the

flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features[15].

Hepatoprotective activity

Hepatoprotective activity of several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities. Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. glutamate-cysteine ligase catalytic subunit (Gclc) expression, glutathione, and ROS levels are reported to be decreased in liver of diabetic mice. Anthocyanins have drawn increasing attention because of their preventive effect against various diseases demonstrated that anthocyanin cyanidin-3-O-β- glucoside (C3G) increases hepatic Gclc expression by increasing cAMP levels to activate protein kinase A (PKA), which Antibacterial Activity. Flavonoids are known to be synthesized by plants in response to microbial infection; thus, it should not be surprising that they have been found invitro to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid rich plant extracts from different species have been reported to possess antibacterial activity. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity[16].

Anti-Inflammatory Activity

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells, release of ROS, RNS, and proinflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders[17].

Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemo preventive agents. Consumption of onions and/or apples, two major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop

cancer of the lung, endometrium, oesophagus, stomach, and colon. The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods[18].

Major molecular mechanisms of action of flavonoids are given as follows:

- Downregulation of mutant p53 protein
- Cell cycle arrest
- Tyrosine kinase inhibition
- Inhibition of heat shock proteins
- Estrogen receptor binding capacity
- Inhibition of expression of Ras proteins

Antiviral Activity

Natural compounds are an important source for the discovery and the development of novel antiviral drugs because of their availability and expected low side effects. Naturally occurring flavonoids with antiviral activity have been recognized since the 1940s and many reports on the antiviral activity of various flavonoids are available. Search of effective drug against human immune deficiency virus (HIV) is the need of hour. Most of the work related with antiviral compounds revolves around inhibition of various enzymes associated with the life cycle of viruses. Structure function relationship between flavonoids and their enzyme inhibitory activity has been observed. Gerdin and Srensso demonstrated that flavan-3-o1 was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2, and similar immunodeficiency virus infections. Baicalin, a flavonoid isolated from Scutellaria baicalensis (Lamieaceae), inhibits HIV-1 infection and replication[19].

Cardioprotective activity

Recent, flavonoids have been stimulated by the potential health benefits arising from the antioxidant activity of those ploy phenolic compounds. The results of their high propensity to transfer electrons, chelate ferrous ions, and scavenge reactive oxygen species. Because of these properties, flavonoids have been considered as potential protectors against chronic cardiotoxicity caused by the cytostatic drug doxorubicin. Doxorubicin is a very effective antitumor agent but its clinical use is limited by the occurrence of a cumulative dose-related cardiotoxicity, resulting in, for example, congestive heart failure (negative inotropic effect) In a recent report, the

cardiotoxicity of doxorubicin on the mouse left atrium has been inhibited by flavonoids, 7- monohydroxyethylrutoside and 7,3,4-trihydroxyethylrutoside[20].

Antiulcer activity

Flavonoids possess anti-ulcerogenic activity. Flavonoid glycosides decreased ulcer and inhibited gastric acid and pepsin secretions. Quercetin, rutin, and kaempferolare used for the treatment of peptic ulcer.[20-23]

Endocrine effects of flavonoids

Flavonoids exhibit hormone like activities. They show similar action to oestrogen and other steroid hormones and are referred to as phytoestrogens. Genistein, daidzein and equal are used for treatment of chronic diseases such as hormone-dependent cancer, cardiovascular disorders and osteoporosis. Genistein is the most promising compound to prevent postmenopausal bone loss in women. Flavonoids are also known to exhibit anti-thyroid effects in animals and humans. Genistein help in the maintenance of insulin, thyroid hormones, adrenocorticotropic hormone, cortisol and corticosterone level, as well as lipid metabolic changes[24].

Drug discovery and development

Drug discovery and development is a research process that identifies a new chemical. Drug discovery is the area of research in which chemo-informatics has found the greatest application. Traditional pharmaceutical industry would require 12-14 years and costing up to $1.2 - $1.4 billion to bring a drug from discovery to market, in this approach drugs were discovered by synthesizing compounds in a time-consuming multi-step processes with failures attributed to poor pharmacokinetics (39%), lack of efficacy (30%), animal toxicity (11%), adverse effects in humans (10%) and various commercial and miscellaneous factors[25].

Today, the process of drug discovery has been revolutionized with the advent of genomics, proteomics, bioinformatics and efficient technologies like, combinatorial chemistry, cheminformatics, high throughput screening (HTS), virtual screening, *de novo* design, *in vitro*, *in silico* ADMET screening, Quantitative structure-activity relationship (QSAR) and structure-based drug design[25].

The process of finding a new drug against a chosen target for a particular disease usually involves high-throughput screening (HTS), wherein large libraries of

chemicals are tested for their ability to modify the target. The primary application of cheminformatics is in the storage of information related to the drug molecules and the efficient presentation of such stored information during the process of lead optimization[25].

A range of parameters can be used to assess the quality of a compound, or a series of compounds, as proposed in the Lipinski's Rule of Five. Such parameters include calculated properties such as C Log P to estimate lipophilicity, rotatable bonds to estimate molecular flexibility, molecular weight, Hydrogen Bond Acceptors and Hydrogen Bond Donors to estimate Pharmacophoric Properties, polar surface area and measured properties, such as potency, in-vitro measurement of enzymatic clearance etc. Some descriptors such as ligand efficiency (LE) and lipophilic efficiency (LiPE) combine such parameters to assess drug likeness[26].

Importance of molecular docking

Pharmaceutical research has successfully incorporated a wealth of molecular modelling methods, within a variety of drug discovery programs, to study complex biological and chemical systems. The integration of computational and experimental strategies has been of great value in the identification and development of novel promising compounds. Broadly used in modern drug design, molecular docking methods explore the ligand conformations adopted within the binding sites of macromolecular targets. This approach also estimates the ligand-receptor binding free energy by evaluating critical phenomena involved in the intermolecular recognition process[25].

Molecular docking has become an increasingly important tool for drug discovery. Flexible receptor molecular docking approaches, especially those including backbone flexibility in receptors, are a challenge for available docking methods. A recently developed Local Move Monte Carlo (LMMC) based approach is introduced as a potential solution to flexible receptor docking problems. Three application examples of molecular docking approaches for drug discovery are provided[25].

The molecular docking approach can be used to model the interaction between a small molecule and a protein at the atomic level, which allow us to

characterize the behavior of small molecules in the binding site of target proteins as well as to elucidate fundamental biochemical processes[25].

Knowing the location of the binding site before docking processes significantly increases the docking efficiency. In many cases, the binding site is indeed known before docking ligands into it. Also, one can obtain information about the sites by comparison of the target protein with a family of proteins sharing a similar function or with proteins co-crystallized with other ligands. In the absence of knowledge about the binding sites, cavity detection programs or online servers, e.g. GRID[27,28], POCKET[29], SurfNet[30,31], PASS[32] and MMC[33] can be utilized to identify putative active sites within proteins. Docking without any assumption about the binding site is called blind docking[25].

Theory of docking

Essentially, the aim of molecular docking is to give a prediction of the ligand-receptor complex structure using computation methods. Docking can be achieved through two interrelated steps: first by sampling conformations of the ligand in the active site of the protein; then ranking these conformations via a scoring function. Ideally, sampling algorithms should be able to reproduce the experimental binding mode and the scoring function should also rank it highest among all generated conformations. From these two perspectives, we give a brief overview of basic docking theory[25].

Molecular Docking

Molecular docking is one of the most frequently used methods in SBDD because of its ability to predict, with a substantial degree of accuracy, the conformation of small-molecule ligands within the appropriate target binding site. Following the development of the first algorithms in the1980s, molecular docking became an essential tool in drug discovery. For example, investigations involving crucial molecular events, including ligand binding modes and the corresponding intermolecular interactions that stabilize the ligand-receptor complex, can be conveniently performed. Furthermore, molecular docking algorithms execute quantitative predictions of binding energetics, providing rankings of docked compounds based on the binding affinity of ligand-receptor complexes[25].

The docking process involves two basic steps:

- Prediction of the ligand conformation as well as its position and orientation within the sites.
- Assessment of the binding affinity.

The early elucidation for the ligand-receptor binding mechanism is the lock-and-key theory[37] in which the ligand fits into the receptor like lock and key. The earliest reported docking methods were based on this theory and both the ligand and receptor were treated as rigid bodies accordingly.

Then the "induced-fit" theory created by Koshland takes the lock-and-key theory a step further, stating that the active site of the protein is continually reshaped by interactions with the ligands as the ligands interact with the protein. This theory suggests that the ligand and receptor should be treated as flexible during docking. Consequently, it could describe the binding events more accurately than the rigid treatment[35].

Lipinski's rule of Five

This rule was postulated by Christopher A. Lipinski in 1997, based on the observation that most of the drugs are relatively small and lipophilic in nature. Lipinski's rule of five is a rule of thumb to evaluate druglikeness[26]. This rule states that, an orally active "drug-like" molecule has:

- Partition coefficient (log P) less than 5
- Molecular weight under 500 daltons
- Not more than 10 hydrogen bond acceptors (O and Ngroup)
- Not more than 5 hydrogen bond donors (OH and NH group)
- Number of violations less than 5
- All the numbers should be multiples of 5, which is the basis for the rules name.

The rule describes about pharmacokinetic properties of a drug in the human body, including their absorption, distribution, metabolism, and excretion (ADME). But this rule does not predict if a compound is pharmacologically active or not.[38] This set of rules suggests that the necessary properties for good oral bioavailability and reflects the notion that pharmacokinetics, toxicity and other adverse effects are directly linked to the chemical structure of a drug[27].

Types of docking[35,36]

Rigid docking

The rigid docking is suitable position for the ligand in receptor environment obtained while maintaining its rigidity.

Flexible docking

In this process, receptor-ligand interaction was obtained by changing internal torsions of ligand into the active site while receptor remains fixed.

Docking approaches

Two approaches mainly popular in molecular docking.

- **Shape complementarity:** This technique is used to describe the matching of ligand and protein as complementarity surfaces.
- **Simulation:** It is the actual docking process additionally calculating the interaction energies between ligand and protein molecule.

Mechanics of docking

To perform a docking screen, the first requirement is a structure of a protein. Protein structure has been determined by using x-ray crystallography or NMR spectroscopy. The protein structure and database of potential ligands serve as inputs to a docking programme. The success of docking program depends on: search algorithm and scoring function[35,36].

Search algorithm

A strict search algorithm would completely elucidate all possible binding modes between ligand and receptor. Various searching algorithms have been developed and widely used in molecular docking software. But it would be too expensive to computationally generate all the possible conformations. Some commonly used searching algorithms are: Monte Carlo (MC) methods, fragment based method, distance geometry, matching method, ligand fit method, point complementarity method, blind docking, inverse docking, genetic algorithms and molecular dynamics[35,36].

Monte Carlo (MC) method

Methods are among the most established and widely used stochastic

optimization techniques. These methods use sampling technique and are able to generate states of low energy conformations. The system makes random moves and accepts or rejects each conformation based on Boltzmann probability. Simulated annealing is a generalization of a Monte Carlo method for examining the equations of state and frozen states of n-body systems. The initial state of the system has random thermal motion within a specified potential force field. The effective temperature of the system (the degree of random motion) is decreased overtime, until a final stable docked position is obtained. The random motion of the ligand allows for exploration of the local search space, and the decreasing temperature of the system acts to drive it to a minimum energy. One of the most widely used simulated annealing procedures is the Metropolis Monte Carlo simulated annealing algorithm[35,36].

Fragment based method

Fragment based methods can be described as dividing the ligand into separate protons or fragments, docking the fragments and finally linking these fragments together with target protein. Some mainly used fragment based methods are Flexx[37].

Distance geometry

Many types of structural information can be expressed as intra or intermolecular distances. The distance geometry formalism allows these distances to be assembled and 3 dimensional structures consistent with them to be calculated. The fast sampling of the conformational space do not always results in reliable results. An example of a program using distance geometry in docking problem is Dock It.[37]

Matching method

This method focuses on complementarity. Ligand atom is placed at the 'best' position in the site, generating a ligand receptor configuration that may require optimization.[37]

Ligand fit method

Ligand fit term provide a rapid accurate protocol for docking small molecules ligand into protein active sites for considering shape complimentarity between ligand and protein active sites.[37]

Point complementarity method

These methods are based on evaluating a shape and/or chemical complementarity between interacting molecule.[37]

Blind docking

It is introduced for detection of possible binding sites and modes of peptide ligand by scanning the entire surface of protein targets.[37]

Inverse docking

This method uses computer for finding toxicity and side effects of protein targets on a small molecule. Knowledge of these targets combined with that of proteomics pharmacokinetic profile can facilitates the assessment of potential toxicities side effect of drug candidate. One of these protocols is selected for docking studies of particular ligand.[37]

Genetic algorithm (GA)

It is adaptive heuristic search technique premised on the evolutionary ideas of natural selection and genetics. The basic concept of GA is designed to simulate processes in natural system necessary for evolution, which is akin to the principles first laid down by Darwin. As such they represent an intelligent exploitation of a random search within a defined search space to solve a problem. In a genetic algorithm, there is a population of solutions that undergo mutation and crossover transformations. The newly generated solutions undergo selection, biased towards the fit among them. The algorithm maintains a selective pressure towards an optimal solution, with a randomized information exchange permitting exploration of the search space. A range of programs implements GA for docking[37].

Molecular dynamics (MD)

Molecular dynamics (MD) is widely used as a powerful simulation method in many fields of molecular modeling. In the context of docking, by moving each atom separately in the field of the rest atoms, MD simulation represents the flexibility of both the ligand and protein more effectively than other algorithms. The disadvantage of MD simulations is that they progress in very small steps and thus have difficulties in stepping over high energy conformational barriers, which may lead to inadequate

sampling. MD simulations are often efficient at local optimization. Thus, a current strategy is to use random search in order to identify the conformation of the ligand, followed by the further subtle MD simulations.[38]

Scoring functions

The purpose of the scoring function is to delineate the correct poses from incorrect poses, or binders from inactive compounds in a reasonable computation time. Scoring functions involve estimating, rather than calculating the binding affinity between the protein and ligand and through these functions, adopting various assumptions and simplifications[38]. Scoring functions can be divided into:

i. Force-field-based scoring functions
ii. Empirical based scoring functions
iii. Knowledge-based scoring functions.

Force-field-based scoring functions

Classical Force-field-based scoring functions assess the binding energy by calculating the sum of the non-bonded (electrostatics and van der Waals) interactions. The electrostatic terms are calculated by a Columbic formulation. Force-field-based scoring functions also have the problem of slow computational speed. Thus cut-off distance is used to handle the non-bonded interactions. This also results in decreasing the accuracy of long-range effects involved in binding[38].

Extensions of force-field-based scoring functions consider the hydrogen bonds, solvation's and entropy contributions. Software programs, such as DOCK, GOLD and AutoDock, offer users such functions. They have some differences in the treatment of hydrogen bonds, the form of the energy function etc. The results of docking with force-field-based functions can be further refined with other techniques, such as linear interaction energy and free-energy perturbation methods (FEP) to improve the accuracy in predicting binding energies[38].

Empirical scoring functions

In empirical scoring functions, binding energy decomposes into several energy components, such as hydrogen bond, ionic interaction, hydrophobic effect and binding entropy. Each component is multiplied by a coefficient and then summed up to give a final score. Coefficients are obtained from regression analysis fitted to a test set of

ligand-protein complexes with known binding affinities. Empirical scoring functions may be treated in a different manner by different software, and the numbers of the terms included are also different. LUDI, PLP, Chem Score are examples derived from empirical scoring functions[38].

Knowledge-based scoring functions

Knowledge based scoring functions use statistical analysis of ligand-protein complexes crystal structures to obtain the inter-atomic contact frequencies and distances between the ligand and protein. The scoring is done by statistically observing the intermolecular relation between the ligand and the biological target protein using "Potential of Mean Force". The intermolecular interactions are mainly taken into account for the functional group or atoms that occur frequently. The result of this method is evaluated based on the binding interactions. PMF, Drug Score, SMoG and Bleep are examples of knowledge-based functions[38].

Various docking software's

Over 60 docking software systems and more than 30 scoring functions are reported. Molecular docking is implemented as part of software packages for molecule design and simulation. More than one search method and scoring functions are provided in order to increase the accuracy of the simulations. Only some of the software was made available and a limited number of them are widely used[35].

DOCK

DOCK is one of the oldest and best-known ligand-protein docking programs. It systematically describes the geometries of ligands and binding sites by sets of spheres, attempting to fit each compound from a database into the binding site and the spheres could be overlapped by means of an approximate clique-detection procedure. The initial version used rigid ligands and edibility was later incorporated via incremental construction of the ligand in the binding pocket of the target protein. In the recent version of DOCK, steric matching-scores with electrostatic and molecular mechanics interaction energies are considered for the ligand-receptor complex. In scoring, the docked orientations of atomic hydrophobicity descriptors are being considered[35].

GOLD (Genetic Optimization for Ligand Docking)

GOLD uses a flexible docking mode for small molecules into protein binding

site which utilizes genetic algorithm for the conformational search that forms a powerful tool for screening and identification of novel lead compounds. It provides docking of flexible ligand and protein with flexible hydroxyl groups. Otherwise the protein is considered to be rigid. This makes it a good choice when the binding pocket contains amino acids that form hydrogen bonds with the ligand. Gold is very highly regarded within the molecular modeling community for its accuracy and reliability and its genetic algorithm parameters are optimized for wide range of virtual screening applications. GOLD has one of the most comprehensive validation test sets and is also available for use at CSC[35,37].

FlexX (Future Leaders Exchange)

A pose-clustering algorithm is used to classify the docked ligand conformers, where the placement of rigid core fragment is based on interaction geometry between fragment and receptor groups. Prior to docking, FlexX cuts the ligand at rotatable bonds into pieces, places a base fragment into the active site, and incrementally builds up the ligand again, using the other pieces. For a protein with known three-dimensional structures and a small ligand molecule, FlexX predicts the geometry of the protein-ligand complex and estimates the binding affinity. It has a bit lower hit rate than DOCK but provides better estimates of Root Mean Square Distance for compounds with correctly predicted binding mode. There is an extension of FlexX called with flexible receptors which has shown to produce better results with significantly lower running times[35,37].

GLIDE (Grid Based Ligand Docking with Energetics)

GLIDE approximates a close and complete systematic search for the conformational, orientation and positional space of the docked ligand. Glide uses a series of hierarchical filters to search for possible locations of the ligand in the active-site region of the receptor. A grid representation for the shape and properties of the receptor is used that progressively scores for ligand posing[35].

SLIDE (Screening for Ligands by Induced-fit Docking)

SLIDE represents a general approach to organic and peptidyl database screening. It can handle large binding-site templates and uses multi-stage indexing to identify feasible subsets of template points for ligand docking. An optimization

approach based on mean-field theory is applied to model induced-fit complementarities, balancing flexibility between the ligand and the protein side chains. SLIDE can screen 100,000 compounds within a few days and returns a ranked list of stearically feasible ligand candidates, ranked by complementarities to the protein's binding site[35].

FRED

FRED takes a multi-conformer library or database of one or more ligands, a target protein structure, a box defining the active site of the protein and several optional parameters. Various options are available for optimization with respect to the built-in scoring functions: optimization of hydroxyl group rotamers, rigid body optimization, torsion optimization, and reduction of the number of poses that are passed on to the next scoring function. The ligand conformers and protein structure are treated as rigid during the majority of the docking process. FRED's docking strategy is to exhaustively score all possible positions of each ligand in the active site. The exhaustive search is based on rigid rotations and translations of each conformer. This novel strategy completely avoids the sampling issues associated with stochastic methods used by most other docking programs (such as Gold, FlexX, Dock and Glide). FRED jobs can also be easily distributed over multiple processors to further reduce docking time[35].

Hammerhead

Hammerhead is suitable for screening large databases of flexible molecules by binding to a protein of known structure. It precisely docks a variety of known flexible ligands, and it spends an average of only a few seconds on each compound during a screen. The approach is completely automated, from the elucidation of protein binding sites, through the docking of molecules, to the final selection of compounds for assay[35].

AutoDock

AutoDock is a script driven flexible automated and random search docking technique operated by altering the ligand or a subset of ligand with several rotatable bonds to predict the binding interaction between small molecules to the known receptor three-dimensional structure. The robustness of AutoDock can be attributed to the Monte Carlo simulated annealing, evolutionary, genetic and Lamarckian genetic

algorithm methods. The prediction of bound conformations such as enzyme-inhibitor complexes, peptide-antibody complexes and even protein-protein interactions has shown a great success through AutoDock. Possible orientations are evaluated with AMBER force field model in conjunction with free energy scoring functions and a large set of protein-ligand complexes with known protein-ligand constants. The newest unreleased version 4 contains side chain flexibility. AutoDock has more informative web pages than its competitors; because of its free academic license[35].

Autodock 4.2

Auto Dock 4.2 uses a semi-empirical free energy force field to evaluate conformations during docking simulations. The force field evaluates binding in two steps. The ligand and protein start in an unbound conformation. In the first step, the intra molecular energies are estimated for the transition from these unbound states to the conformation of the ligand and protein in the bound state. The second step then evaluates the intermolecular energies of combining the ligand and protein in their bound conformations. The parameters are based on the amber force field[32].

Applications of drug design

- Lead identification
- Lead optimization
- Structural elucidation by X-ray crystallography
- Virtual screening analysis
- Structure based drug design
- Combinatorial library design
- Chemical mechanistic studies

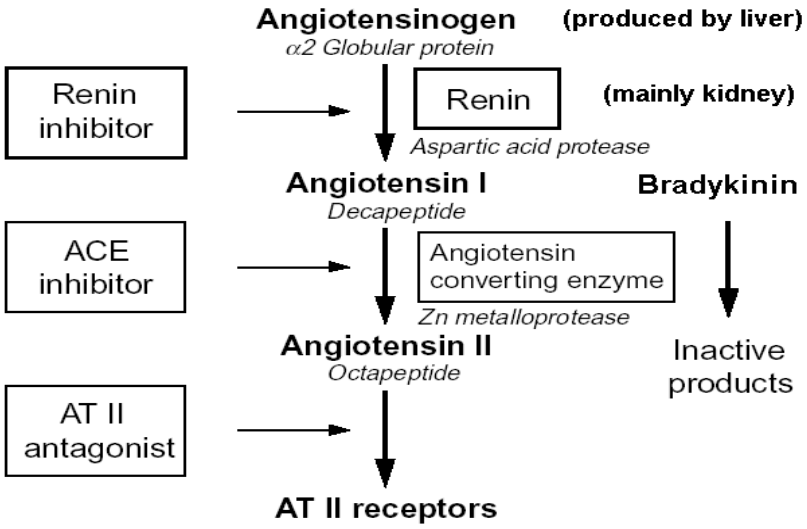


Fig 5: Biosynthesis of angiotensin converting enzyme

Angiotensin converting enzyme

Angiotensin converting enzyme is a zinc metalo-peptidase that hydrolyses carboxyl terminal dipeptide of many oligopeptide substrates, inclusive of angiotensin I (Ang I) and bradykinin. This enzyme is most commonly related with the control of blood pressure.[2,16] The octapeptide Angiotensin II is one of the most potent vasoconstrictors. ACE is widely distributed in various body tissues, in which predominantly present in kidney epithelium.[16] In Renin – Angiotensin Aldosterone system (RAS), ACE and angiotensin II are biologically active components of RAS. These components play a key role in maintenance of blood pressure. ACE inhibitors competitively inhibit the angiotensin converting enzyme which is used to treat hypertension, cardiac failure, diabetic nephropathy, acute myocardial infarction[39].

Doxorubicin induced myocardial infarction in rats

Doxorubicin, also known as Adriamycin, is an anthracycline antibiotic, that was discovered from a mutated strain of *Streptomyces peucetius*. It has been used against cancer since 1960s. It is one of the most effective anticancer drugs, because of its cardiotoxicity doxorubicin have been reported. Therefore, chemotherapy with doxorubicin is limited by its cardiotoxicity. The development of cumulative dose-dependent cardiomyopathy may occur many years after the cessation of doxorubicin treatment. It has been calculated that approximately 10% of patients treated with doxorubicin will develop cardiomyopathy and heart failure. Owing to the importance of doxorubicin in the chemotherapy treatment for many cancer types, strategies have

been tried to prevent or attenuate the side effects of doxorubicin administration, including the use of doxorubicin analogues, alternative drug-delivery methods, and iron-chelating agents. However, so far, the ability of treatments to prevent or attenuate doxorubicin-induced damage has been limited, and discovery of novel agents for reducing its side effects is still needed[40].

Doxorubicin consists of a naphthacene quinone nucleus and daunosamine, an amino sugar. Doxorubicin has both hydrophilic and hydrophobic regions, allowing it to bind to plasma proteins as well as cell membranes. Doxorubicin is also amphoteric; in having both acidic and basic functions. It is these features that make doxorubicin a versatile compound, allowing it to enter various cellular compartments. Doxorubicin can be reduced intracellularly into doxorubicinol and this metabolite also has biological activity. Doxorubicin can also be reduced to a semiquinone radical by many of the intracellular oxidoreductases. Reoxidation of this radical resulted in the production of reactive oxygen. Doxorubicin operates on several levels by different molecular mechanisms including an interaction with iron, upsetting calcium homeostasis, altering the activity of intracellular or intra mitochondrial oxidant enzymes, and binding to topoisomerases promoting their dysfunction[40].

In silico Docking of angiotensin converting enzyme

Muhammad and Fatima., 2015 analysed the inhibitory action of quercetin by computational docking studies. They have isolated quercetin from buckwheat and onion and used as ligand for molecular interaction. The crystallographic structure of molecular target angiotensin-converting enzyme (ACE) (peptidyl-dipeptidase A) was obtained from PDB database (PDB ID: 1O86). Enalapril, a ACE inhibitor was taken as the standard for comparative analysis. Computational docking analysis was performed using PyRx, AutoDock Vina option based on scoring functions. The quercetin showed optimum binding affinity with a molecular target (angiotensin-converting-enzyme) with the binding energy of −8.5 kcal/mol as compared to the standard (-7.0 kcal/mol). These results indicated that quercetin could be one of the potential ligands to treat hypertension, myocardial infarction, and congestive heart failure.

Hafeez *et al*., 2014 [42] worked on molecular modelling of plant flavonoids as angiotensin converting enzyme (ACE) inhibitors in hypertension. In their study, molecular modelling results clearly demonstrated that flavonoids have a similar binding sites and interactions with ACE as those of the synthetic drugs and prove that dietary flavonoids may possess properties of blood pressure regulation. In terms of the mode of action, flavonoids had shown competitive type of inhibition for ACE. Thus, *in silico* study is actually an added advantage to screen the ACE inhibition and flavonoids may serve as useful leads for the synthesis of clinically useful antihypertensive drugs.

Shafari et al., 2016 [43] investigated flavonoid rich *Orthosiphon stamineous* extract was a new candidate for ACE-I inhibition. In this study, they demonstrated the changes in the flavonoidal active core affects its capacity to inhibit the ACE and production of chelate with zn (II) ion and this interaction stabilized by other interactions with amino acids in the active site.

Synthesis of flavonoids

Kshathriya *et al.*, 2013 [44] designed and synthesized a class of flavonoids based on the backbone of 2-phenylchromen-4-one. Flavones are mainly found in cereals and herbs. Flavones are biologically active compounds. Therefore, number of synthetic methods were developed. Some of the well-known methods used for synthesis of flavones are Baker & Venkatraman synthesis and Claisen-Schmidt condensation.

Tang *et al.*, 2004 [45] described a novel approach to the synthesize 6-amino-7-hydroxyflavone. Reaction in acetone 2, 4-dihydroxy-5-nitroacetophenone and benzoyl chloride in the presence potassium carbonate affords 3-benzoyl-7-hydroxy-6-nitroflavone, which is cleaved in 5% ethanolic potassium hydroxide to give 1-(2,4-dihydroxy-5-nitrophenyl)-3-phenyl-1,3-propanedione. The 1,3-diketone thus formed is then transformed into 7-hydroxy-6-nitroflavone, followed by reduction to afford the title compound.

Patil., 2013[69] synthesized 3- benzoyl flavone 4 via modified Baker-Venkatraman reaction. The chemical structure of the newly synthesized compound was confirmed by 1H NMR, MS, IR spectral data. Acute oral toxicity of the synthesized compound was determined as per OECD guidelines-423 and dose for biological activity was found to be 200 mg/ kg body weight. The compound was screened for anti-inflammatory (carrageenan-induced rat paw oedema model), anti-oxidant activity (DPPH-radical scavenging model) and brine shrimp cyto-toxicity assay. On screening, it was found that 3-benzoyl flavone with electron donating substitutions has significant antioxidant potential and anti-inflammatory activity. Also screening of anti-cancer activity on 60 cell lines of 9 different types of cancers by NCI – NIH showed that compound is active against renal cancer (cell line UO-31). Results obtained from pharmacological screening is satisfactory as compared with corresponding standards used. Collectively, these results show that flavonoid with electron donating substitution like dimethyl amino [$-N(CH_3)_2$] group has significant antioxidant activity. The strong antioxidant potential could allow this flavone derivative to be administered for prevention of numerous free radical based diseases.

Huang *et al.*,2003[60] designed and synthesized the flavonoids of *scutellaria baicalensis*. They successfully attained an extremely efficient route for the preparation of baicalein, oroxylin A, and wogonin. To our best knowledge, for total synthesis of these three pharmacologically diversified flavonoids, our approach is the only practical path featuring in beginning with a common starting material, using affordable reagents and proceeding under mild conditions and thus suitable for largescale pilot-plant synthesis. Various flavone derivatives are now being prepared in our laboratory by the above-mentioned methodology with a view to extensively evaluating their biological activities. The experimental details and biological data will be published shortly.

***In vitro* angiotensin converting enzyme inhibitory activity**

Ranjini *et al.*, 2015[80] examined aqueous extracts of *Rauwolfia serpentina* leaves and *Allium sativum* cloves on sheep kidney and lung ACE. In their method Hippuryl-Histidyl-Leucine (HHL) was used as substrate, sheep kidney and lung ACE activity was measured and the hippuric acid released was measured spectrophotometrically at 228 nm. The results of linearity of ACE activity in kidney and lung was established with HHL as substrate for the incubation period of 30 min at 37°C. ACE activity was confirmed with specific ACE inhibitors like captopril, lisinopril and enalapril. About 25 µl of *R. serpentina* leaf extract reduced ACE activity by 68% and 57% in kidney and lung respectively and 25 µl of *A. sativum* cloves extract reduced ACE activity by 50% and 60% in kidney and lung respectively. The significant inhibition of kidney and lung ACE activity by these two plant products suggests their possible role in controlling blood pressure as a mode of treatment for cardiovascular diseases, when used as supplement medicine.

Balasuriya *et al*., 2011 extracted flavonoids from the apple skin and the major constituents of the extract and some of the selected metabolites were assessed for *in vitro* ACE inhibitory property. All the flavonoids tested have a potential to inhibit ACE *in vitro* and inhibitory property varies according to the type of sugar moiety attached at the C_3 position.

Deo *et al*., 2016 reported the potential of selected Australian medicinal plant extracts to inhibit protein glycation and enzymes related to hyperglycaemia and

hypertension. Extracts of the leaves of *Petalostigma banksii* and *P. pubescens* showed the strongest inhibition of α-amylase with IC50 values of 166.50 ± 5.50 µg/mL and 160.20 ± 27.92 µg/mL, respectively. The *P. pubescens* leaf extract was also the strongest inhibitor of α-glucosidase with an IC50 of 167.83 ± 23.82 µg/mL. Testing for the antiglycation potential of the extracts, measured as inhibition of formation of protein-bound fluorescent AGEs, showed that *P. banksii* root and fruit extracts had IC50 values of 34.49 ± 4.31 µg/mL and 47.72 ± 1.65 µg/mL, respectively, which were significantly lower ($p < 0.05$) than other extracts. The inhibitory effect on α-amylase, α-glucosidase and the antiglycation potential of the extracts did not correlate with the total phenolic, total flavonoid, FRAP or DPPH. For ACE inhibition, IC50 values ranged between 266.27 ± 6.91 to 695.17 ± 15.38 µg/mL.

Doxorubicin induced myocardial infarction

Chen *et al.*, 2016[55] studied the protective effects of water and ethanol extracts of three varieties of yam, against DOX-induced cardiotoxicity in experimental mice. DOX treatment led to significant decreases in the ratio of heart weight to body weight and heart rate, and increases in blood pressure and the serum level of lactate dehydrogenase, a marker of cardiotoxicity, were recovered by yam extracts, especially in water extract. Yam extracts also decreased the cardiac levels of thiobarbituric acid reactive substances, reactive oxygen species, and inflammatory factors, as well as the expression of nuclear factor kappa B, while ethanol extracts of two species were shown to be more potent. Moreover, yam extracts had a role in increasing the activities of glutathione peroxidase and superoxide dismutase, thus improving the DOX-induced alterations in oxidative status in the heart tissue of DOX-treated mice. All ethanol extracts of yam exhibited their antiapoptotic abilities on caspase-3 activation and mitochondrial dysfunction. Based on these findings, it was concluded that yam has significant cardioprotective properties against DOX-induced damage via its multiple effects on antioxidant, anti-inflammatory, or antiapoptotic activities.

Koti *et al.*, 2012[62] explored Vedic Guard, a polyherbal formulation used in the treatment of various ailments, however, is not scientifically assessed for its effect on doxorubicin-induced cardiotoxicity. Cardiotoxicity was induced by doxorubicin.

Vedic guard was administered as pre-treatment for 2 weeks and then for 2 weeks alternated with doxorubicin. The general observations, mortality, histopathology, biomarker like lactate dehydrogenase, creatine phosphokinase, aspartate aminotransferase, alanine transaminase, electrocardiographic parameters, antioxidants such as glutathione, superoxide dismutase, and catalase were monitored after 3 weeks of last dose. The repeated administration of DXR causes cardiomyopathy associated with an antioxidant deficit. Pre-treatment with Vedic guard decreases serum enzymes levels to that of normal values. Vedic guard significantly protected the myocardium from the toxic effect of doxorubicin, by increasing the levels of antioxidants such as GSH, SOD, and CAT and decreased the elevated level of malondialdehyde. It also reduced the severity of cellular damage of the myocardium confirmed by histopathology. The results of the present study indicated **c**ardioprotective effect of Vedic guard might be attributed to its antioxidant activity.

Davey *et al*.,2011[83] proved the inotropic and cardioprotective effect of *Terminalia paniculata* in doxorubicin induced cardiotoxicity in rats. *Terminalia paniculata* extract reduced mortality and mitigation of cardiac injury markers, improved ATPase activity and restoration of oxidant/ antioxidant status as well as lessening histopathological changes. The study proved that *Terminalia paniculata* improved cardiac protection against doxorubicin cardiotoxicity.

Swami *et al*., 2011 studied the preventive role of curcumin against doxorubicin- induced myocardial toxicity in rats. The general observations, mortality, histopathology, biomarker enzymes like lactate dehydrogenase, creatine phosphokinase, biochemical parameters such as aspartate aminotransferase alanine aminotransferase and alkaline phosphatase, antioxidant enzymes such as glutathione, superoxide dismutase and catalase were monitored after three weeks of last dose. The repeated administration of Dox induced cardiomyopathy associated with an antioxidant deficit and increased level of biomarkers. Pre-treatment with the curcumin significantly protected myocardium from the toxic effects of Dox by reducing the elevated level of biomarker enzymes and biochemical parameters back to normal. Curcumin increased the reduced level of GSH, SOD and CAT and decreased the

elevated level of malondialdehyde in cardiac tissue. The biochemical and histopathology reports support the cardioprotective effect of curcumin which could be attributed to antioxidant.

Vijay *et al*., 2011 examined the cardioprotective, antioxidant activities and phytochemical analysis of *Gmelina arborea* on doxorubicin induced myocardial necrosis on rats. Doxorubicin generates free oxygen radicals and resulted in serious dose-limiting cardiotoxicity. Supplementations with *Gmelina arborea* proved effective in reducing oxidative stress associated with several ailments. GA protected against DOX-induced MI and increased the levels of marker enzymes. The extract significantly inhibited glutathione (GSH) depletion in cardiac tissues. The reductions of cardiac activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) were significantly mitigated. Pre-treatment of GA significantly guarded against DOX-induced rise of serum lactate dehydrogenase (LDH). GA alleviated histopathological changes in rats' hearts treated with DOX.

Palani et al., 2012 examined the cardioprotective activity of ethanol extract of *Flacourtia indica* (FI) against doxorubicin (DOX)-induced myocardial infarction(MI) in rats. Different phytoconstituents were identified by gas chromatography, mass spectroscopy. DOX is a chemotherapeutic agent which produces free oxygen radicals that result in serious dose-limiting cardiotoxicity. The pre-treatment with test drug to DOX-treated rats significantly prevented the altered biochemical parameters such as serum marker enzymes serum glutamate-pyruvate transaminase, serum glutamate oxaloacetate transaminase, creatine phosphokinase, and lactate dehydrogenase, lipid profile such as low-density lipoprotein, very low-density lipoprotein, triglycerides, high-density lipoprotein, total cholesterol, and antioxidant parameters such as superoxide dismutase, glutathione, catalase, glutathione peroxidase, and malondialdehyde to near normal level. Serum urea, uric acid, and alkaline phosphate which are increased on DOX administration registered near normal values on pre-treatment with FI. The data obtained suggested that the ethanol extract of FI can

prevent heart damage by DOX-induced MI in rats and this is likely mediated through its antioxidant activities.

Thippeswamy *et al*., 2011 investigated the effect of the aqueous extract of *Phyllanthus niruri* against doxorubicin-induced myocardial toxicity in rats. Cardiotoxicity was produced by Dox administration and test drug was administered as pre-treatment for 2 weeks alternated with Dox for the next 2 weeks. The general observations, mortality, histopathology, biomarker enzymes like lactate dehydrogenase (LDH), creatinine phosphokinase (CPK) and alkaline phosphatase, diagnostic enzyme markers like aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and antioxidants such as glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were monitored after 3 weeks of the last dose. Pre-treatment with aqueous extract of *Phyllanthus niruri* significantly protected the myocardium from the toxic effects of Dox by reducing the elevated level of biomarker and diagnostic enzymes like LDH, CPK, AST and ALT to the normal levels. Aqueous extract increased the GSH, SOD and CAT levels and decreased the MDA levels in cardiac tissue. Administration of Dox caused cardiomyopathy associated with an antioxidant deficiency. These results suggest a cardioprotective effect of *P. niruri* due to its antioxidant properties.

Swamy *et al*., 2011 explored the preventive and curative role of ascorbic acid on doxorubicin-induced myocardial toxicity in rats. The test drug was given for 15 days and doxorubicin administered in accumulative dose. The biochemical parameters such as tissue glutathione, malondialdehyde, catalase, and superoxide dismutase, and enzyme biomarkers such as creatine phosphokinase, lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were monitored. Pre-treatment with ascorbic acid significantly protected the myocardium from the toxic effect of dox, by increasing the levels of antioxidant enzymes toward normal and decreased the levels of MDA, CPK, LDH, AST, and ALT as compared with dox-treated rats. Post-treatment with ascorbic acid to dox-treated group significantly increased the levels of tissue GSH, SOD, CAT and significantly decreased the level of MDA as compared with dox-treated group. It also reduced the severity of cellular damage of the

myocardium as confirmed by histopathology. The restoration of the endogenous antioxidant system clearly depicts that ascorbic acid produced its protective effect by scavenging the reactive oxygen species. The results obtained in this study provided evidence for the usefulness of the ascorbic acid as a cardioprotective agent.

Warpe *et al*., 2014 revealed the cardioprotective activity of ellagic acid on doxorubicin induced cardiotoxicity. Ellagic acid was administered respectively once daily orally for 6 weeks. On the last day of the study, blood was collected by retro-orbital puncture and LDH and CK-MB were estimated. The animals were cannulated and the ECG and hemodynamic parameters were recorded. The animals were then sacrificed and histology of heart was performed. Doxorubicin showed cardiotoxicity manifested by changes in serum marker enzymes, ECG and hemodynamic parameters which was further confirmed by histology of heart. This doxorubicin induced changes were attenuated by treatment with ellagic acid. Ellagic acid treatment for 6 weeks protects the heart of rats in doxorubicin induced cardiotoxicity.

Sun *et al*., 2013 explored the possible protective effect of isorhamnetin against Dox-induced cardiotoxicity and its underlying mechanisms. Rats were intraperitoneally (i.p.) administered with Dox to duplicate the model of Dox-induced chronic cardiotoxicity. Daily pre-treatment with isorhamnetin (5 mg/kg, i.p.) for 7 days was found to reduce Dox-induced myocardial damage significantly, including the decline of cardiac index, decreased in the release of serum cardiac enzymes and amelioration of heart vacuolation. *In vitro* studies on H9c2 cardiomyocytes, isorhamnetin was effective to reduce Dox-induced cell toxicity. A further mechanism study indicated that isorhamnetin pre-treatment can counteract Dox-induced oxidative stress and suppress the activation of mitochondrion apoptotic pathway and mitogen-activated protein kinase pathway. Isorhamnetin also potentiated the anti-cancer activity of Dox in MCF-7, HepG2 and Hep2 cells. These findings indicated that isorhamnetin can be used as an adjuvant therapy for the long-term clinical use of Dox.

Acker *et al*., 1995 investigated whether 7-monohydroxyethylrutoside (mono HER), a powerful antioxidative flavonoid with extremely low toxicity, can provide protection to an extent comparable to the clinically successful cardioxane (ICRF-187).

Balb/c mice of 20-25 g were equipped i.p. with a telemeter to measure ECG. They were given six i.v. doses of doxorubicin (4 mg kg-) at weekly intervals. ICRF-187 (50 mg kg) or mono HER (500 mg kg'-) were administered i.p. 1 h before doxorubicin administration. In the 2 mono HER groups the treatment continued with either 1 or 4 additional injections per week. A saline and mono HER treated group served as controls. After these 6 weeks, they were observed for another 2 weeks. At the end of this study (week 8) the ST interval had increased by 16.7 ± 2.7 ms in doxorubicin-treated mice. At that time, the ST interval had increased by only 1.8 0.9 ms in ICRF-187co-mediated mice and in mono HER co-medicated mice by only 1.7 + 0.8 and 5.1 + 1.7 ms (5- and 2-dayschedule, respectively, all P <0.001 relative to doxorubicin and not significantly different from control). The ECG of the control animals did not change during the entire study. The QRS complex did not change in either group. it can be concluded that mono HER protects against doxorubicin-induced cardiotoxicity and merits further evaluation in this respect.

Kumar *et al*., 2016 designed to evaluate the cardioprotective potential of *Acorus calamus* on doxorubicin-induced myocardial toxicity in *Albino Wistar* rats were used in this study. DOX was administered intraperitoneally in six equal injections (each containing 2.5 mg/kg to a total cumulative dose of 15 mg/kg over 2 weeks to produce cardiotoxicity. Control and treated group animals were used for the estimation of lipid and cardiac biomarkers. Animals were sacrificed and heart tissue was used for the assay of glutathione (GSH), malondialdehyde(MDA), catalase (CAT), and superoxide dismutase (SOD) and also for the membrane-bound enzymes namely Na^+-K^+-ATPase, Ca^+2-ATPase, and Mg^+2-ATPase. The remaining portion of the heart tissue was used for histopathological studies. Pre-treatment with drug decreases serum enzyme levels and lipid profiles brought to the near normal values, and it significantly protected the myocardium from the toxic effect of DOX, by increasing the levels of antioxidant enzymes such as GSH, SOD, and CAT toward normal and decreasing the increased level of MDA. AC (200 mg/kg) + DOX showed significant increase in Na^+-K^+-ATPase and Mg^+2-ATPase and decrease in Ca^+2-ATPase compared to DOX-

treated groups. The results of this study indicated that the cardioprotective effect of AC might be attributed to its antioxidant property.

Razmaraii *et al*., 2016 determined the ability of grape seed extract as a powerful antioxidant in preventing adverse effect of doxorubicin (DOX) on heart function. Left ventricular function and hemodynamic parameters were assessed using echocardiography, electrocardiography and a Millar pressure catheter. Histopathological analysis and *in vitro* antitumor activity were also evaluated. DOX induced heart damage in rats through decreasing the left ventricular systolic and diastolic pressures, rate of rise/decrease of LV pressure, ejection fraction, fractional shortening and contractility index as demonstrated by echocardiography, electrocardiography and hemodynamic parameters relative to control group. Our data demonstrated that GSE treatment markedly attenuated DOX-induced toxicity, structural changes in myocardium and improved ventricular function. Additionally, GSE did not intervene with the antitumor effect of DOX. The results suggest that GSE is potentially protective against DOX-induced toxicity in rat heart and maybe increase therapeutic index of DOX in human cancer treatment.

Sattar *et al*., 2012 investigated cardioprotective effect of *Stachys schimperi* Vatke on DOX-induced cardiotoxicity in rats. The phenolic profile of the methanol extract was determined qualitatively by HPLC. The methanol extract showed moderate protection against DOX-induced alteration in cardiac oxidative stress markers; GSH and MDA, and cardiac serum markers; CK-MB and LDH activities. Additionally, histopathological study denoted mild protection against DOX-induced cardiotoxicity.

Kulkarni and Swamy, 2015 studied the protective and potential effect of gallic acid against doxorubicin (DOX) induced cardiotoxicity. Doxorubicin administered intraperitoneally in six equal injections to total cumulative doses of 15 mg/kg over a period of 2 weeks to induce cardiotoxicity. The estimation of cardiac and lipid biomarkers of both control and treated animals were observed. *In vivo* antioxidant parameters such as glutathione, superoxide dismutase, catalase, and malondialdehyde were also monitored. The remaining portion of the heart tissue used for histopathological studies. The treatment with doxorubicin increased the levels of

serum cardiac and lipid biomarker which were brought down by gallic acid treatment. It also exhibited electrocardiographic changes such as reduced R wave and ST segment elevation. Histopathological study revealed that gallic acid restored the myocardial cells towards normal. The restoration of the endogenous antioxidant system clearly depicts that gallic acid have produced its protective effect by scavenging the reactive oxygen species. The results of this study indicated that the cardioprotective effect of gallic acid might be attributed to its antioxidant property.

Karim *et al.*, 2001 revealed the protective effects of enalapril, losartan, vitamin A, aspirin, melatonin and a combination of melatonin and aspirin against doxorubicin induced cardiotoxicity in rats. Enalapril, losartan, vitamin A, melatonin and melatonin + aspirin were given for 5 days to albino rats followed by doxorubicin. Biochemical parameters like serum AST and blood GSH, tissue TBARS and GSH were estimated to assess to cardiac function. Doxorubicin treatment increased the levels of serum AST in rats. Pre-treatment with enalapril, losartan, vitamin A, aspirin, melatonin, and melatonin + aspirin decreased the levels of serum AST of the rats treated with doxorubicin. Doxorubicin induced fall in blood GSH, tissue GSH were reversed by enalapril, losartan, vitamin A, aspirin, melatonin and melatonin + aspirin. Doxorubicin induced elevation of TBARS in cardiac tissue was also significantly reduced by the above treatments. Combined administration of melatonin and aspirin may offer better protection as compared to melatonin alone against doxorubicin induced cardiotoxicity.

Abirami and Kanagavalli, 2013 evaluated doxorubicin induced cardiotoxicity in rats using grape seed proanthocyanidin. Intraperitoneal injection of DOX [10mg kg^{-1}/b.wt] administrated once a day for 15 days was revealed by elevated serum creatine phoshokinase [CPK], lactate dehydrogenase [LDH], aspartate transaminase [ASP], alanine transaminase [ALT], HDL cholesterol and triglycerides in comparison of control and associated with increasing levels of myocardial malondialdehyde [MDA] with simultaneously decrease in the level of glutathione peroxidase [Gpx], glutathione-S transferase [GST] and glutathione reductase [GR] daily oral administration of aqueous suspension of GSP seeds extract. The study showed that GSP seeds possess antioxidant and cardioprotective effect.

OBJECTIVE OF THE STUDY

Myocardial infarction (MI) is a leading cause of mortality, it is the irreversible necrosis of heart muscles secondary to prolonged ischemia. This usually results from an imbalance of oxygen supply and demand. The incidence of MI is rising in young individuals owing to change in lifestyle pattern, eating habits, more stress and workload. Incidence remained highest in Males as compared to females. Highly associated risk factor in young myocardial infarction patients are dyslipidaemia, smoking, diabetes, hypertension.

Angiotensin Converting Enzyme (ACE), the central component of the renin - angiotensin system (RAS) controls the blood pressure by regulating the volume of fluids in the body. It converts the hormone angiotensin-I to the angiotensin- II. Therefore, ACE indirectly increases blood pressure by causing blood vessels to constrict.

The inhibition of ACE is considered as one of the most effective therapeutic strategy for the treatment of myocardial infarction. Enzyme inhibitors are used as potent therapeutic agents for the treatment of various diseases. More than 100 drugs are used worldwide as enzyme inhibitors. ACE inhibitors such as captopril, enalapril, fosinopril and Ramipril are currently available in the market.

Flavonoids are fairly versatile compounds and are easy to synthesize. They are associated with a wide range of pharmacological actions including antioxidant, antimicrobial, anticancer, anti-inflammatory activities and also possesses ACE inhibitory activity.

Doxorubicin is a powerful, well-established and highly efficacious drug in many kinds of cancers. But its clinical usefulness is still restricted due to its specific toxicities to cardiac tissues. Congestive heartfailure, cardiomayopathy and electrocardiographic changes were demonstrated after cumulative doxorubicin administration. Myocardial infarction produced by doxorubicin through different mechanisms like lipid peroxidation, mitochondrial DNA damage, myocardial injury,

contractile dysfunction, apoptosis. The flavonoids having an ability to inhibit these mechanisms.

The present study mainly focuses on the design and synthesis of certain flavonoid derivatives in order to explore the extent of their cardioprotective activity and also identify the conformations of ligands in the active site of protein and to predict the affinity of the ligands towards the protein. Drug discovery tools have been utilized in designing new chemical entities which are safe and effective without consuming much of the research hours.

PLAN OF WORK

- ✓ **STEP I**

  Designing and determination of ACE inhibitory activity of 50 flavonoid compounds
- ✓ **STEP II**

  Determination of bioactivity scores by using molinspiration software
- ✓ **STEP III**

  To carry out the molecular docking studies using Autodock 4.2 based on the bioactive score.
- ✓ **STEP IV**

  Evaluation of toxicity using the software T.E.S.T
- ✓ **STEP V**

  Selection of five flavonoids based on their docking score and toxicity values
- ✓ **STEP VI**

  Synthesis, purification and spectral analysis of 5 flavonoids
- ✓ **STEP VII**

  In vitro screening of five synthesized flavonoids for ACE inhibitory activity
- ✓ **STEP VIII**

  Acute toxicity study of two selected synthesized flavonoids based on their *in vitro* results
- ✓ **STEP IX**

  In vivo screening of the two flavonoids by doxorubicin induced myocardial infarction in rats
- ✓ **STEP X**

  Euthanised and collection of blood by retro-orbital plexus puncture method for biochemical estimations
- ✓ **STEP XI**

  Dissection and preparation of tissue homogenate using heart for biochemical estimations
- ✓ **STEP XII**

  Section of the heart tissue used for histopathology
- ✓ **STEPXIII**

  Tabulation, compilation of results and statistical analysis of data obtained

MATERIALS AND METHODS

EVALUATION OF DRUG LIKENESS PROPERTIES

The pharmacologically active substituents are characterized by calculating steric, hydrophobic, electronic, and hydrogen bonding properties as well as by the drug-likeness score. The theory of drug-likeness score helps to optimize pharmaceutical and pharmacokinetic properties, for example, chemical stability, solubility, distribution profile and bioavailability. The molecular descriptors have developed as rationally predictive and informative, for example, the Lipinski's Rule-of-Five. The better oral absorption of the ligands and drug likeness scores are constructed by getting information about the solubility, diffusion, Log P, molecular weight etc. Molinspiration software is used to evaluate the Lipinski's rule of five.

Lipinski's rule of five calculations

1. Open the molinspiration home page.
2. For calculating using molinspiration it requires JAVA in the computer.
3. Click calculation of molecular properties of drug likeness
4. Draw the structure of flavonoids in the active window.
5. Click and calculate properties.
6. Save the properties.

Evaluation of ADMET properties

The ADMET studies were performed using Accerlys Accord for excel. From this absorption, distribution, metabolism, of the selected ligands was evaluated. The 2D structure was directly introduced into Accord for excel to carry out ADMET screening by using ChemSketch software. The data for descriptors are blood brain barrier (BBB), plasma protein binding, aqueous solubility and hepatotoxicity. After loading the structure through the function model various descriptors are tabulated.

IN SILICO DOCKING AND BINDING INTERACTION STUDIES

SOFTWARES AND DATA BASES USED

- Accerlys discovery studio viewer 4.0.1
- Molinspiration
- RCSB protein data bank
- OnlineSMILES translator

- MGL tools-
    - AutoDock 4.2
    - Python 2.7 molecule viewer 1.5.6
    - Vision 1.5.6
    - Cygwin 64
- ChemSketch
- T.E.S.T version 4.2.1
- MEDCHEM designer

In silico docking study on angiotensin converting enzyme using AutoDock 4.2

STEP I:

Ligand file format conversion

- Flavonoid structures are drawn in CHEMSKETCH.
- Tools→generate→SMILES notation
- Copy the smile notation and upload the smiles in online smile translator-cactus.nci.nih.gov/services/translate
- By choosing the required file format and save the file as pdb format

STEP II:

Protein structure refinement

Angiotensin Converting Enzyme (PDB ID:1086) download from RCSB (Research Co-laboratory for Structural Bioinformatics) Protein Data Bank and the protein should be refined before use for docking.

- Open Accelrys discovery studio viewer
- File→open→RCSBPDB file
- View→ hierarchy→click water molecules→ select all water molecules→cut
- Select ligand, which is unnecessary and cut
- Save the molecule in a desired location

STEP III:

Docking with AutoDock 4.2

- Open the saved refined protein from the location as in pdb format
- Preparation of target and ligand in AUTODOCK 4.2

STEP IV:

Preparation of protein

- AUTODOCK 4.2 → File→ Read molecule→ Choose refined enzyme file

Elimination of water molecule carried out by;

- Select→ Select from string→ Residue (*HOH*) → Add→ Dismiss
- Edit→ Hydrogen→ Add→ Polar only→ Ok
- Edit→charges→ Add kollmann charges→ Ok
- File→save→ Write pdb→ Browse→Save→ Ok
- Edit→ Delete all molecules→ Continue

STEP V:

Preparation of ligand

- Ligand→input→open
- Ligand→torsion tree→detect root
- Ligand→ torsion tree→show root expansion
- Ligand→ torsion tree→choose torsions→done
- Ligand→ torsion tree→set number of torsions→dismiss
- Ligand→ torsion tree→hide root expansion
- Ligand→ torsion tree→show/hide root marker
- Ligand→output→ save as pdbqt file
- Edit→delete→delete all molecules→ continue

Conversion of pdb files of protein in to pdbqt file

- Grid→ Macromolecule→ Open→ Save as pdbqt

AutoGrid calculation and creating "gpf" file

- Grid→set map types→ open ligand
- Grid→grid box→set 60 points in XYZ
- File→close saving current
- Grid→output→save as GPF
- Edit→delete→delete all molecules→continue

AUTODOCK calculation and creating 'dpf" file

- Docking→macromolecule→set rigid file name →open
- Docking→ligand→open→accept
- Docking→search parameters→genetic algorith→accept
- Docking→docking parameters→accept
- Docking→output→lamarckian genetic algorithm→save as dpf

Programming of 'Auto Grid' and 'Auto Dock' execution

Open Cygwin and type as given below:

- cd C:
- cd cygwin
- cd usr
- cd local
- cd bin
- ls

program should list out the pdb, pdbqt, gpf and dpf files of an enzyme and ligand molecule.

Then type as:

- ./autogrid4.exe<space>–p<space>ligand.gpf-l<space> ligand.glg

If a ligand gets into the spacing of the grid, then the execution of this command will be;

- ✓ '*Successful completion'*

Then type as:

- ./autodock4.exe<space> –p<space> ligand.dpf-l<space> ligand.dlg

If the ligand binds to the amino acids through 10 different conformations, then the execution of this command will be;

- ✓ *Successful completion'*

STEP IV:

Viewing docking results

Reading the docking log file. dlg

- Toggle the AutoDock Tools button
- Analyse → Docking
- Analyse → Conformations → Load
- Double click on the conformation for to view it

Visualizing docked conformations

- Analyse → Dockings → Play
- Load dlg file
- Choose the suitable conformations
- Analyse → Docking → Show Interactions

Obtaining snap shots of docked pose

- File → Read Molecule

- Analyse→ Dockings → Open dlg file
- Analyse→ Macromolecule → Choose pdbqt file.
- Analyse→ Conformations → Load
- Double click the desired conformation
- Analyse → Docking → Show Interactions

Proteins and ligand interaction will be displayed. Zoom it and increase the contrast by holding right key and ctrl. Rapid energy evaluation was attained by pre-calculating the atomic resemblance potentials for each atom in the selected flavonoids. In the AutoGrid process, the target was enclosed on a three-dimensional grid point and the energy of interface of each atom in the flavonoids were encountered. The following docking factors were chosen for the Lamarckian genetic algorithm as follows: population size of 150 individuals, 2.5 million energy evaluations, maximum of 27000 generations, and number of top individuals to automatically survive to next generation of 1, mutation rate of 0.02, crossover rate of 0.8, 10 docking runs, and random initial positions and conformations. The probability of performing local search on a single compound in the population was set to 0.06. AutoDock was run various times to obtain various docked conformations, and used to calculate the predicted binding energy[46].

CHEMICALS AND REAGENTS

2-Hydroxy acetophenone, dry acetone, anhydrous potassium carbonate, dilute hydrochloric acid, 2-ethoxy benzoic acid, 2,4-dichloro benzoic acid 2-chlorobenzoic acid, 4-chlorobenzoic acid, 2-chloro 5-nitro benzoic acid were procured from Sigma Aldrich. Sheep lung tissue was purchased from slaughter house at Coimbatore. Hippuryl- L- Histidyl - L- Leucine (HHL) (Sigma Aldrich), enalapril (Dr.Reddys,Hyderabad), doxorubicin (Pfizer New York), borate buffer pH-8.3, bovine serum albumin (Himedia), alkaline copper sulphate, Folin-Ciocalteau reagent, HEPES buffer, cyanuric chloride, 1 4-dioxan, EDTA, doxorubicin, enalapril, alkaline copper solution, Tris- HCl buffer, TCA-TBA-HCl reagent, Sodium azide, phenazonium methosulphate, glutathione, Ellman's reagent, all serum parameter kits are procured from agappe diagnostics, Kerala.

INSTRUMENTS / EQUIPMENTS USED

Semi auto-analyzer (Agappe Diagnostics Ltd, Mumbai), Centrifuge (Remi Instruments Ltd., Kolkata), UV- Visible Spectrophotometer (JASCO V-630 spectrophotometer), MR-VIS- visual melting range apparatus.

SYNTHESIS OF FLAVONOIDS

To a stirred solution of O-hydroxy acetophenone (0.3moles) in dry acetone and anhydrous potassium carbonate was added and stirred at room temperature for 10min. The substituted benzoyl chloride (0.9moles) added in small portion to the reaction mixture and stirred for 30min. The reaction mixture was then refluxed for 24hrs.The solvent was removed by distillation under reduced pressure and the resultant solution was acidified with dilute hydrochloric acid. The product thus obtained was filtered, dried and recrystallized from ethanol[13].

Table 1: IUPAC NAMES OF THE SYNTHESIZED FLAVONOIDS

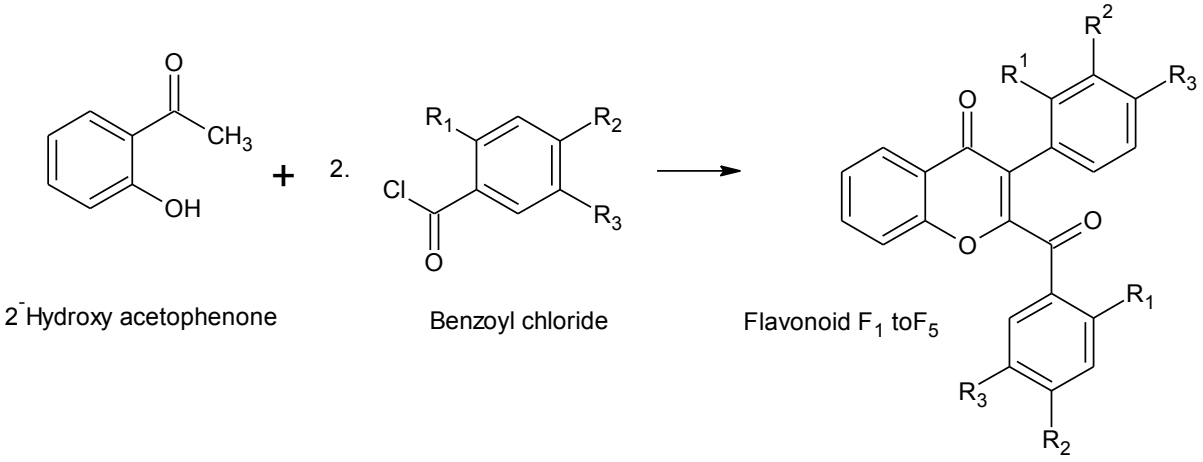
COMPOUNDS	IUPAC NAMES
F1	3-(2-ethoxy benzoyl)-2-(2-ethoxy phenyl)-4H-chromen-4-one
F2	3-(2,4-dichloro benzoyl)-2-(2,4-dichloro phenyl)-4H-chromen-4-one
F3	3-(2-chloro benzoyl)-2-(2-chloro phenyl)-4H-chromen-4-one
F4	3-(4-chloro benzoyl)-2-(4-chloro phenyl)-4H-chromen-4-one
F5	3-(2-chloro-5-nitro benzoyl)-2-(2-chloro-5-nitro phenyl)-4H-chromen-4-one

Table 2: PHYSICAL DATA OF SYNTHESIZED FLAVONOIDS

COMPOUNDS	MOLECULAR FORMULA	MOLECULAR WEIGHT	MELTING POINT
F1	$C_{26}H_{22}O_5$	414.44	150.5°C
F2	$C_{22}H_{10}Cl_4O_3$	464.12	210.5°C
F3	$C_{22}H_{12}Cl_2O_3$	395.23	113.5°C
F4	$C_{22}H_{12}Cl_2O_3$	395.23	235.5°C
F5	$C_{22}H_{10}Cl_2N_2O_7$	485.23	123.5°C

COMPOUND CODE	R_1	R_2	R_3
F1	Cl	H	H
F2	H	Cl	H
F3	OC_2H_5	H	H
F4	Cl	Cl	H
F5	Cl	H	NO_2

Fig 6: Scheme for the synthesis of flavonoids



DETERMINATION OF MELTING POINT

The melting point of the synthesized compounds (F1-F5) was determined by using open capillary tube using MR-VIS visual melting range apparatus.

***IN VITRO* ANGIOTENSIN CONVERTING ENZYME INHITORY ASSAY**

Isolation of angiotensin converting enzyme from sheep lung extract

Sheep lung was collected from slaughter house and one gram of sheep lung tissue was sliced and homogenised in 10ml of the ice cold 100Mm borate buffer (pH8.3) containing 50mM KCl using a homogeniser at 4°C. the homogenate was centrifuged at 8000g at 4°C for half an hour, and the supernatant was collected. Remove, if any impurities present and stored at 4°C. this supernatant was used as the source of ACE[14].

Estimation of protein content by Lowry method

Different dilutions of Bovine serum albumin (1.2-1µg/ml) were prepared from stock BSA solution (1mg/ml) in a test tube. The final volume in each test tube was adjusted to 5ml with distilled water. From these dilutions, pipette out 0.2ml protein

solution to different test tubes and add 2ml of alkaline copper sulphate reagent (analytical reagent) and mix well. The solution was incubated for 10min at room temperature. Then add 0.2ml of Folin Ciocalteau solution to each test tube and incubated for 30min. The colorimeter was set to zero with blank and optical density was measured at 450nm. Plot the absorbance against protein concentration to get a standard calibration curve. Check the concentration of unknown from the standard curve.

Estimation of ace from sheep lung extract

ACE inhibitory was measured by using Hippuryl-L-histidyl-leucine (HHL) as substrate. This reaction mixture contained 0.2ml of 5mM HHL prepared in 200mM borate buffer (pH8.3) with 1000 mM KCl. The reaction was initiated by the addition of different concentration of lung extract and then incubated at 37°C for 30min. the reaction was stopped by the addition of 2ml of HEPES buffer and 1ml of 136mM cyanuric chloride in 1,4-dioxan and yellow colour was developed was measured at 450 nm. Various concentration of hippuric acid (which is formed by the reaction of ACE on HHL) were used for the preparation of standard graph.

***In vitro* angiotensin converting enzyme inhibitory activity**

Colorimetric method

The reaction mixture contained 5mM HHL prepared in 200mM Borate buffer (Ph8.3), containing 1000mM KCl 10µl of different concentration of unknown drug sample (50-800µg), lung extract (50µl) and distilled water in a volume of 1ml. The reaction mixtures were incubated at 37°c for 30min. the reaction was stopped by adding 2ml of HEPES buffer (pH9), which contain 2.5mM EDTA. Finally add 1ml of 136mM cyanuric chloride in 1,4-dioxan was added to the reaction mixture and shake vigorously for 15sec. The absorbance of yellow colour developed was measured at 405nm. Enalapril was used as the standard drug for comparison with the assay system. (Rangini *et al.,*2015)

$$\% \text{ ACE inhibition} = \frac{(A0 - A1)}{A0} \times \mathbf{100}$$

A_0= Absorbance of the control

A_1= Absorbance of the standard/compounds

EXPERIMENTAL ANIMALS

Healthy male *Wistar* rats weighing 220g±20%, procured from Kerala Veterinary and Animal Science University, Thrissur. The study protocol was approved by the Institutional Animal Ethical Committee and all procedures were performed in accordance with the recommendations for the proper care and use of laboratory animals[48].

Housing and feeding conditions

The animals were kept in cages under ambient temperature (22 ± 3°C) with 12 h light/dark cycle. They were fed with standard rat laboratory diet water *ad libitum*.

ACUTE TOXICITY STUDY OF FLAVONOIDS

Acute oral toxicity testing was carried out in accordance with the OECD guideline 420 Acute Oral Toxicity – Fixed Dose Procedure method (OECD, 2002).

Procedure

The acute toxicity study was done by two steps - sighting study and main study. The purpose of the sighting study is to allow selection of the appropriate starting dose for the main study. Healthy adult female (generally slightly more sensitive than male) *Wistar* rats weighing between 220±20%g body weight was procured and kept in cages under ambient temperature (22±3°C) with 12 h light/dark cycle. The animals were randomly selected, marked and kept in their cages for 5 days prior to dosing for acclimatization to laboratory conditions. The animals were fasted over-night and were provided with water *ad libitum*. The synthesized test compounds (flavonoids) was suspended in 0.5% CMC. Totally 18 animals were used for this study. Sighting study was conducted for two compounds at dose levels of 5, 50, 300 and 2000 mg/kg body weight using 8 animals and if the animal dosed with 2000 mg/kg *b.w*. survived without any toxic manifestations during the sighting study and the same dose was selected for the main study. The main study was conducted for 2 compounds at dose of 2000 mg/kg body weight using 10 animals. After the administration of the flavonoids, food was withheld for further 3-4 hours.

Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Once daily cage side observations included changes in skin and fur, eyes and mucous membrane (nasal), and also respiratory rate,

circulatory (heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (drowsiness, gait, tremors and convulsions) changes.

Table 4: Design of acute toxicity studies

Study involved	Dose (mg/kg)	No. of animals
Sighting study	5	2
	50	2
	300	2
	2000	2
Main study	2000	10

IN VIVO DOXORUBICIN- INDUCED MYOCARDIAL INFARCTION

Animals were divided into 5 groups of 8 animals each. The values for control and negative control group (doxorubicin 10 mg/kg i.p) were taken from archives based on the recommendations of CPCSEA-IAEC and denoted as group I and II respectively. Group III animals received flavonoid I- low dose (100 mg/kg) and doxorubicin 10 mg/kg i.p on 26th day. Group IV, flavonoid I-high dose (200 mg/kg) and doxorubicin 10 mg/kg ip on 26th day. Group V served as flavonoid II- low dose (100 mg/kg) and doxorubicin 10 mg/kg i.p on 26th day. Group VI, flavonoid II-high dose (200 mg/kg) and doxorubicin 10 mg/kg i.p on 26th day. Group VII animals received Enalapril (10 mg/kg) as the standard and doxorubicin 10 mg/kg i.p on 26th day[48].

Dissection and homogenisation

On 28th day of the treatment i.e., 48 hours after doxorubicin administration, animals were anaesthetized with diethyl ether. The blood was collected through retro-orbital plexus method and the serum was separated for biochemical estimations. The rats were sacrificed by euthanasia and heart was separated immediately, weighed, immersed in saline and dissected for histopathological examination[48].

Serum biochemical parameters

The following are the biochemical parameters used CK-MB, LDH, TROPONIN-I, SGPT/ALT, SGOT/AST, ALP, triglycerides, total cholesterol, HDL, LDL

CK-MB

Principle: The procedure involves measurement of CK activity in the presence of an antibody to CK-M monomer. This antibody completely inhibits the activity of CK-MM and half of the activity of CK-MB, while not affecting the B subunit activity of CK-MB and CK-BB. Then we use CK method to quantitatively determine CK-B activity. The CK-MB activity is obtained by multiplying the CK-B activity by two.

Procedure: Mix the 4 volumes of Reagent 1(R1: imidazole-125mmol/l, D- glucose-25mmol/l, N-Acetyl-L-Cysteine- 25mmol/l, Magnesium acetate- 12.5mmol/l, NADP-2.52mmol/l, EDTA- 2.02mmol/l, Hexokinase >6800 U/L, Anti human polyclonal CK-M antibody (sheep) sufficient to inhibit up to 2000U/L of CK-MM) with 1 volume of reagent 2 (R2: Creatine phosphate-250mmol/l, ADP-15.2mmol/l, AMP-25mmol/l, Diadenosine phosphate- 103mmol/l, G-6-PDH >8800 U/L)[65]

LDH

Principle: Pyruvate is reacted with NADH and one molecule of hydrogen in the presence of lactate dehydrogenase gives L- Lactate and NAD^+

Procedure: Mix 4 volume of Reagent1 (R1: Tris buffer -80mmol/L, Pyruvate-1.6mmol/L) with 1 volume of reagent 2(R2: NADH-240mmol/L)[66]

Troponin I

principle: The HeartScan One Step Troponin I Card Test (Serum/Plasma) is a qualitative, membrane based immunoassay for the detection of troponin I in serum or plasma. The membrane is pre-coated with capture reagent on the test line region of the test. During testing, the serum or plasma specimen reacts with the particle coated with anti-troponin I antibodies. The mixture migrates upward on the membrane chromatographically by capillary action to react with capture reagent on the membrane and generates a coloured line. The presence of this coloured line in the test line region indicates a positive result, while its absence indicates a negative result. To serve as a procedural control, a coloured line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

Procedure:

- Bring the pouch to room temperature before opening it. Remove the test device from the sealed pouch and use it as soon as possible. Best results will be obtained if the test is performed immediately after opening the foil pouch.

- Add 2 drops of serum in to the sample window.
- Wait for coloured lines to appear. Read results at 10 minutes. Do not interpret results after 20 minutes[68].

SGPT

Principle: L-alanine reacts with alpha- ketoglutarate in the presence of ALT gives pyruvate and L-glutamate. Pyruvate reacts with NADH and one molecule of hydrogen ion in the presence of lactate dehydrogenase gives L-lactate and NAD^+

Procedure: Mix 4 volume of Reagent 1 (R1: Tris buffer-110mmol/l, L-Alanine-600mmol/L, LDH >1500U/L) with 1 volume of Reagent 2 (R2: Alpha-Ketoglutarate-16mmol/L, NADH-0.24mmol/L)[69]

SGOT

Principle: L-Aspartate reacts with alpha- ketoglutarate in the presence of AST gives Oxaloacetate and L-Glutamate. Oxaloacetate reacts with NADH and one molecule of hydrogen ion gives L-Malate and NAD^+

Procedure: Mix 4 volume of Reagent 1(R1: Tris Buffer-88mmol/L, L-Aspartate-260mmol/L, LDH >1500 U/L, MDH >900 U/L) with 1volume of Reagent 2 (R2: alpha-ketoglutarate-12mmol/L, NADH-0.24mmol/L)[70]

ALP

Principle: Para-nitrophenyl phosphate reacts with one molecule of water in the presence of ALP gives Para-nitrophenol and inorganic phosphate

Procedure: Mix 4 volume of reagent 1 (R1: Diethanolamine buffer- 125mmol/L, Magnesium chloride-0.625mmol/L,) with 1 volume of Reagent 2 (R2: P-Nitro phenyl phosphate-50mmol/L)[71]

Triglycerides

Principle: Enzymatic determination of triglycerides;

- TGL reacting with water in the presence of lipoprotein lipase to gives Glycerol and Fatty acid.
- Glycerol is reacting with ATP in the presence of glycerol kinase to gives Glycerol -3- phosphate and ADP.
- Glycerol -3- phosphate reacting with 2 molecules of oxygen in the presence of Glycerol -3- phosphate oxidase gives dihydroxyacetophenone and 1 molecule of hydrogen peroxide.

- 2 molecules of hydrogen peroxide reacting with 4- aminoantipyrine and TOPS gives violet coloured complex.

Procedure: Mix the triglyceride reagent (pipes buffer-5mmol/L, TOPS-5.3MMOL/L, Potassium ferrocyanate-10mmol/L, Magnsium salt- 17mmol, 4-aminoantipyrine-0.8mmol/L, ATP-3.15mmol/L, lipoprotein lipase >1800 U/L, Glycerol-3-phosphate oxidase >3500 U/L, Peroxidase >450) with triglycerides standard concentration- 200mg/dL[71]

HDL

Principle: The reaction between cholesterol other than HDL and the enzyme for cholesterol assay is suppressed by the electrostatic interaction between polyanions and cationic substances. Hydrogen peroxide is formed by the free cholesterol in HDL by cholesterol oxidase. Oxidative condensation of EMSE and 4-AA is caused by hydrogen peroxide in the presence of peroxidase, and the absorbance of the resulting red- purple quinone is measured to obtain the cholesterol value in HDL. HDL in the presence of polyanions and cationic substances suppress the reaction with enzyme. HDL (cholesterol esters) reacts with one water molecule in the presence of cholesterol esterase gives HDL (free cholesterol) and fatty acids.

HDL (free cholesterol) reacts with two molecules of oxygen and one molecule of hydrogen ion in the presence of cholesterol oxidase gives cholestenone and hydrogen peroxide. Two molecules of hydrogen peroxide react with 4-AA, EMSE, three molecules of hydrogen and one molecule of oxygen in the presence of peroxidase gives violet quinone and five molecules of water.

Procedure: Mix the reagent-1 (R1: N-ethyl-N-(3-methylphenyl)-N-succinylehyenediame (EMSE) and reagent-2 (R2: cholesterol oxidase 4-aminoantipyrin(4-AA)[72]

LDL

Principle: This assay method uses a surfactant for selectively solubilizing LDL alone in the cholesterol assay system that employs cholesterol esterase and cholesterol oxidase. It passes the ester cholesterol and free cholesterol contained in the LDL to the cholesterol reaction system to determine LDL cholesterol. The enzyme reactions to other, non-LDL lipoproteins (HDL, VLDL, chilomicrons) are inhibited by the surfactant and by the sugar compounds. These lipoproteins are therefore not passed to

the cholesterol reaction system and consequently remain in the reaction liquid as lipoproteins.

Procedure: Mix the reagent-1(R1: HSDA-1mmol/L, good's buffer) and reagent-2 (cholesterol esterase- 2.0U/L, cholesterol oxidase- 1.0mmol/L, 4-Aminoantipyrin-2.5mmol/L and good's buffer)[73]

Preparation of tissue homogenates

The heart tissue was removed and washed immediately with ice-cold saline to remove blood. A 10% w/v heart tissue homogenate was prepared in ice-cold potassium phosphate buffer (100mM, pH 7.4) followed by centrifugation at 5000g for 10 min. the resulting supernatant was used for the estimation of biochemical parameters[77].

Biochemical parameters for heart tissue homogenate

- Estimation of total protein content

Determination of enzymatic antioxidants

- Assay of catalase
- Estimation of glutathione peroxidase (GPx)
- Assay of superoxide dismutase (SOD)
- Estimation of glutathione reductase (GSSH)

Determination of non-enzymatic antioxidants

- Estimation of reduced glutathione (GSH)

Estimation of malondialdehyde (MDA)

Estimation of total protein content

To 0.1ml of heart tissue homogenate, 4.0 ml of alkaline copper solution was added and allowed to stand for 10 min. Then, 0.4 ml of phenol reagent was added very rapidly and mixed quickly and incubated in room temperature for 30 min for colour development. Reading was taken against blank prepared with distilled water at 610 nm in UV-visible spectrophotometer. The protein content was calculated from standard curve prepared with bovine serum albumin and results were expressed as µg/mg heart tissue[79].

Determination of enzymatic antioxidants

Assay of catalase

The reaction mixture contained 2.0 ml of homogenate and 1.0 ml of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0). a system devoid of the substrate (hydrogen peroxide) served as a control. Reaction was started by the addition

of the substrate and decrease in absorbance mentioned at 240 nm for 30 seconds at 25°C. The difference in absorbance per unit time was expressed as the activity and three parallel experiments were conducted. One unit is defined as the amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25°C[81].

Estimation of glutathione peroxidase (GPx)

The reaction mixture consists of 0.2 ml of 0.4 M Tris buffer, 0.1 ml of 1.0 mM sodium azide, 0.1 ml of 0.042% hydrogen peroxide, 0.2 ml of 200 mM glutathione and 0.2 ml of heart tissue homogenate supernatant incubated at 37°C for 10 min. The reaction was arrested by the addition 0.1 ml of 10% TCA and the absorbance was taken at 340 nm. Activity was expressed as nmoles/min/mg heart protein[82].

Assay of superoxide dismutase (SOD)

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml of 186 μM Phenazonium methosulphate (PMS), 0.3 ml of 300 μM nitro blue tetrazolium chloride (NBT), 0.2 ml of 780 μM NADH, 1.0 ml of homogenate and distilled water to a final volume of 3.0 ml. reaction was started by the addition of NADH and incubated at 30°C for 1 min. The reaction was stopped by the addition of 1.0 ml of glacial acetic acid and the mixture was stirred vigorously. 4.0 ml of n-butanol was added to the mixture and shaken well. The mixture was allowed to stand for 10 min, centrifuged, the butanol layer was taken out and absorbance was measured at 560 nm against a butanol blank. A system devoid of enzyme served as the control and three parallel experiments were conducted[83].

Estimation of glutathione reductase (GSSH)

The enzyme activity was determined spectrophotometrically by the decrease in absorbance of NADPH at 340 nm. The reaction mixture contained 2.1 ml of 0.25 mM potassium phosphate buffer pH 7.6, 0.1 ml of 0.001 M NADPH, 0.2 ml of 0.0165 M oxidized glutathione and 0.1 ml of bovine serum albumin (10 mg/ml). The reaction was started by the addition of 0.02 ml of heart tissue homogenate with mixing and the decrease in absorbance at 340 nm was measured for 3 min against a blank. Glutathione reductase activity was expressed as nmoles/min/mg heart protein at 30°C[84].

Determination of non-enzymatic antioxidants

Estimation of reduced glutathione (GSH)

Heart was homogenized in 10% w/v cold 20mM EDTA solution on ice. After deproteinization with 5% TCA, an aliquot of the supernatant was allowed to react with 150 µM DTNB. The product was detected and quantified spectrophotometrically at 416 nm. Pure GSH was used as standard for establishing the calibration curve and three parallel experiments were conducted[85].

Estimation of Lipid peroxidation indices

Estimation of malondialdehyde (MDA)

One ml of the heart tissue homogenate was combined with 2 ml of TCA-TBA-HCl reagent (15% trichloro acetic acid (TCA) and 0.375% thiobarbituric acid (TBA) in 0.25 N HCl) and boiled for 15 min. precipitate was removed after cooling by centrifugation at 1000g for 10 min and absorbance of the sample was read at 535 nm against a blank without tissue homogenate. The levels of MDA were calculated using extinction coefficient calculation. The values are expressed as n moles/min/mg heart tissue[80].

Histopathological studies

After draining the blood heart samples were excised, washed with normal saline and processed with separately for histopathological observations. The portion of the heart were incised and immersed in 10% formalin for 48 hrs for histopathological examination. Paraffin sections were taken at 5µm thickness processed in alcohol-xylene series and was stained with haematoxylin-eosin dye. The sections were examined microscopically for histopathological changes[19].

Statistical analysis

The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post-test using Graph pad. P values <0.05 were considered as statistically significant condition.

RESULTS

IN-SILICO DOCKING STUDIES

Evaluation of drug likeness properties of the flavonoids

In silico predictions of the drug likeness properties are useful in the drug development process of a new chemical entity**.** The drug likeness property of a compound was evaluated based on the Lipinski's Rule of Five. Lipinski's Rule of five allows calculating the oral bioavailability parameters for the compounds used as an oral drug. The parameters such as molecular weight, log P, hydrogen bond donors, and hydrogen bond acceptors of the compound were calculated for the Lipinski's Rule. If the compound shows any violation it cannot be considered for further *in silico* screening studies.

Analysis of the receptor/ligand complex models generated after successful docking of the flavonoids was based on the parameters such as hydrogen bond interactions, π–π interactions, binding energy, RMSD of active site residues and orientation of the docked compound within the active site. As a general rule, in most of the potent anti-inflammatory compounds, both hydrogen bond and π–π hydrophobic interactions between the compound and the active sites of the receptor have been found to be responsible for mediating the biological activity. The possible structural fragments of the flavonoids (about 50 flavonoids) were designed with the help of ChemSketch and they were subjected to test their drug likeness properties with the help of Molinspiration server.

Table 5: List of flavonoids selected for docking studies

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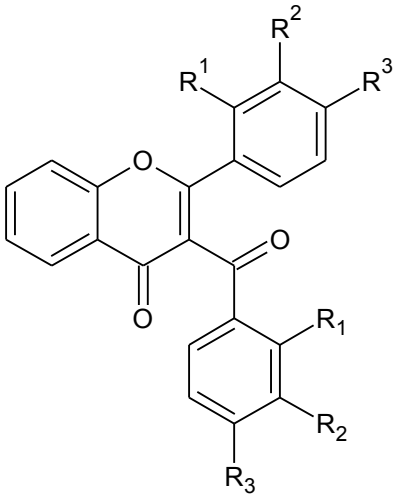
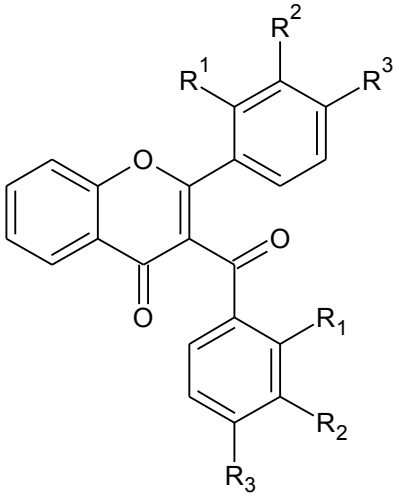
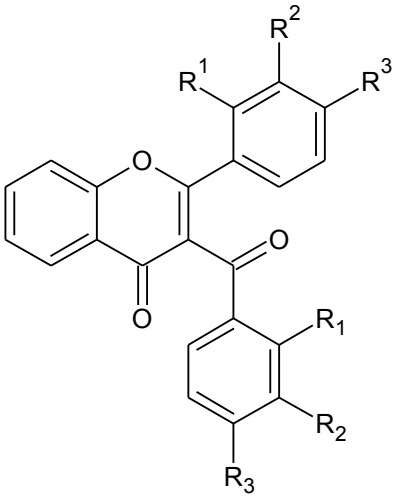
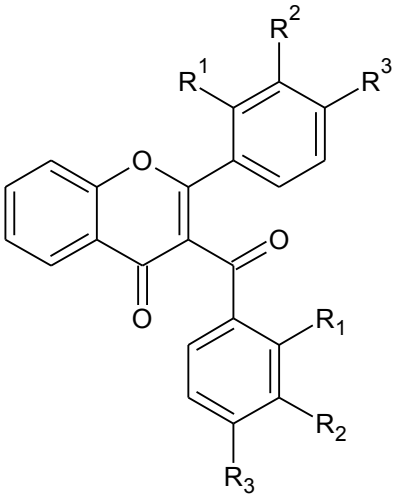
					
1	F1	R_1=Cl, R_2=Cl, R_3=H	12	F12	R_1=Cl, R_2=Br, R_3=H
2	F2	R_1=Cl, R_2=H, R_3=Cl	13	F13	R_1=Cl, R_2=H, R_3=Fl
3	F3	R_1=Cl, R_2=OCH_3, R_3=H	14	F14	R_1=Cl, R_2=H, R_3=NO_2
4	F4	R_1=Cl, R_2=Cl, R_3=Cl	15	F15	R_1=Cl, R_2=H, R_3=OCH
5	F5	R_1=Cl, R_2= Cl, R_3=NO_2	16	F16	R_1=Cl, R_2=H, R_3=CH_3
6	F6	R_1=Cl, R_2=H, R_3=OH	17	F17	R_1=H, R_2=OH, R_3=H
7	F7	R_1=Cl, R_2=H, R_3=NO_2	18	F18	R_1=Cl, R_2=H, R_3=H
8	F8	R_1=H, R_2=H, R_3=Br	19	F19	R_1=Br, R_2=H, R_3=H
9	F9	R_1=H, R_2=H, R_3=CH_3 NH	20	F20	R_1=Cl, R_2=H, R_3=NH_2
10	F10	R_1=Cl, R_2=H, R_3=Cl	21	F21	R_1=OH, R_2=H, R_3=H
11	F11	R_1=Br, R_2=H, R_3=H	22	F22	R_1=H, R_2=H, R_3=Br

Continued

S. No	C code	BASIC NUCLEUS	S. No	C code	BASIC NUCLEUS
					
23	F23	R_1=Cl, R_2=NH_2, R_3=H	34	F34	R_1=CH_3, R_2=H, R_3=H
24	F24	R_1=OH, R_2=H, R_3=Fl	35	F35	R_1=H, R_2=H, R_3=F
25	F25	R_1=OH, R_2=H, R_3=NO_2	36	F36	R_1=H, R_2=H, R_3=NO_2
26	F26	R_1=Cl, R_2=OCH_3, R_3=H	37	F37	R_1=H, R_2=H, R_3=OCH_3
27	F27	R_1=CH_3, R_2=H, R_3=CH_3	38	F38	R_1=, R_2=H, R_3=CH_3
28	F28	R_1=H, R_2=H, R_3=OH	39	F39	R_1=H, R_2=H, R_3=OH
29	F29	R_1=Cl, R_2=H, R_3=NO_2	40	F40	R_1=NO_2, R_2=H, R_3=H
30	F30	R_1=Br, R_2=H, R_3=Br	41	F41	R_1=Cl, R_2=Br, R_3=H
31	F31	R_1=OH, R_2=H, R_3=OH	42	F42	R_1=H, R_2=H, R_3=NH_2CH_3
32	F32	R_1=NO_2, R_2=H, R_3=H	43	F43	R_1=Cl, R_2=H, R_3=Cl
33	F33	R_1=Cl, R_2=NO_2, R_3=H	44	F44	R_1=Br, R_2=H, R_3=Br

Continued

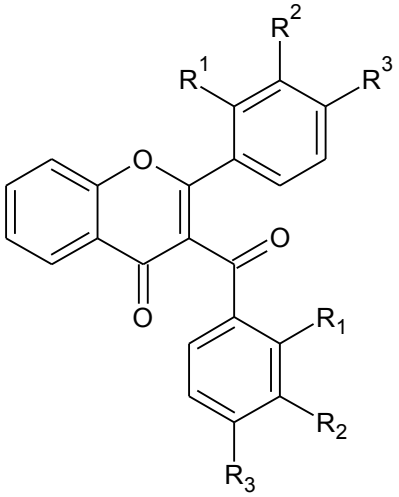
S. No	C code	BASIC NUCLEUS 
45	F45	R_1=Cl, R_2=H, R_3=H
46	F46	R_1=Fl, R_2=H, R_3=H
47	F47	R_1=Cl, R_2=H, R_3=NO_2
48	F48	R_1=Cl, R_2=H, R_3= OCH_3
49	F49	R_1=Cl, R_2=H, R_3=CH_3
50	F50	R_1=Cl, R_2=H, R_3=OH

Table 6: Drug likeness properties of the flavonoids using Molinspiration software

Compound code	Log P	Polar surface area	No. of atoms	No. of hydrogen bond acceptors	No. of hydrogen bond donors	No. of violations	No. of rotatable bonds	Enzyme inhibitor
F1	0.235	47.28	29	3	0	1	1	0.13
F2	2.615	50.13	28	3	0	0	2	0.11
F3	3.198	73.89	23	2	0	1	1	0.23
F4	3.411	31.23	25	2	0	1	1	0.12
F5	2.362	76.03	221	5	0	0	2	0.16
F6	3.709	45.23	19	3	1	0	1	0.11
F7	2.112	30.21	19	2	0	0	1	0.21
F8	3.307	33.45	23	3	0	0	2	0.08
F9	2.103	30.21	70	2	0	0	1	0.21
F10	4.563	43.21	20	3	0	0	2	0.11
F11	5.321	30.21	45	2	0	1	1	0.44
F12	5.180	39.45	19	2	0	1	1	0.22

Compound code	Log P	Polar surface area	No. of atoms	No. of hydrogen bond acceptors	No. of hydrogen bond donors	No. of violations	No. of rotatable bonds	Enzyme inhibitor
F13	5.044	30.21	23	2	0	1	1	0.32
F14	4.455	75.45	22	3	0	0	2	0.14
F15	4.334	30.21	21	5	0	0	2	0.22
F16	5.240	30.21	19	2	0	1	1	0.13
F17	5.017	76.03	19	2	0	1	1	0.09
F18	4.315	45.36	18	5	0	0	2	0.16
F19	4.515	50.44	19	2	0	0	1	0.11
F20	2.118	33.45	19	3	1	0	1	0.36
F21	4.491	30.21	25	3	0	0	2	0.16
F22	4.842	39.45	19	2	0	0	1	0.42
F23	3.187	76.03	21	5	0	0	2	0.31
F24	4.332	76.03	21	5	0	0	2	0.63
F25	2.158	121.86	78	3	0	0	3	0.11
F26	4.480	76.03	21	5	0	0	3	0.24

Compound code	Log P	Polar surface area	No. of atoms	No. of hydrogen bond acceptors	No. of hydrogen bond donors	No. of violations	No. of rotatable bonds	Enzyme inhibitor
F27	4.325	76.03	21	5	0	0	2	0.32
F28	3.623	121.86	23	8	0	0	3	0.12
F29	3.845	76.03	23	5	0	0	2	0.06
F30	3.197	96.26	21	6	1	0	2	0.12
F31	3.772	79.27	23	6	0	0	3	0.13
F32	3.738	85.27	23	6	0	0	3	0.18
F33	4.124	76.03	21	5	0	0	2	0.15
F34	4.95	30.21	19	2	0	0	1	0.12
F35	4.826	30.21	19	2	0	0	1	0.43
F36	4.102	76.03	21	5	0	0	2	0.15
F37	5.23	30.21	19	2	0	0	1	0.25
F38	2.255	30.21	19	2	0	0	1	0.23
F39	4.363	30.21	31	2	0	0	1	0.65
F40	1.684	50.41	19	3	1	0	1	0.32

Compound code	Log P	Polar surface area	No. of atoms	No. of hydrogen bond acceptors	No. of hydrogen bond donors	No. of violations	No. of rotatable bonds	Enzyme inhibitor
F41	4.142	76.03	21	5	0	0	2	0.12
F42	4.268	33.45	21	3	0	0	2	0.21
F43	4.611	30.21	21	2	0	0	1	0.21
F44	4.232	39.45	20	3	0	0	2	0.56
F45	4.042	50.44	19	3	1	0	1	0.35
F46	4.253	50.44	45	3	1	0	1	0.38
F47	5.120	96.26	21	6	1	0	2	0.42
F48	4.023	50.44	19	3	1	0	1	0.13
F49	2.156	50.44	19	3	1	0	1	0.09
F50	4.258	96.26	58	6	1	0	4	0.18

Molecular docking studies

In the present study, all the selected flavonoids showed excellent score which indicates that these compounds have no violations in Lipinski's Rule of 5 (Table). Based on the drug likeness score, the flavonoids were selected for the further prediction of *in silico* biological activity using AutoDock 4.2.

The compounds that have passed the drug likeness properties were considered for molecular docking studies. *In silico* docking study was carried out to identify the inhibiting potential of selected flavonoids against angiotensin converting enzyme (PDB ID: 1086). In this study 50 different flavonoids were selected for the *in silico* docking studies. The docking studies were performed by the use of AutoDock4.2. In the docking studies, if a compound shows lesser binding energy compared to the standard it proves that the compound has higher activity. The docking poses were ranked according to their docking scores and both the ranked list of docked ligands and their corresponding binding poses. Binding energy of the individual compounds were calculated using the following formula,

$$\text{Binding energy} = A+B+C-D$$

Where**,** A denotes the final intermolecular energy + vander Waals energy (vdW) + hydrogen bonds + desolvation energy + electrostatic energy (kcal/mol). B denotes the final total internal energy (kcal/mol). C denotes the torsional free energy (kcal/mol) and D denotes the unbound system energy (kcal/mol).

All the 50 flavonoids showed binding energy ranging between -10.44 kcal/mol to -6.59 kcal/mol. F1 flavonoid showed excellent binding energy -9.50 kcal/mol and standard enalapril -6.59 kcal/mol. F1 flavonoid showed higher binding energy when compared to other synthetic flavonoids. F1-F5 compounds showed excellent binding energy when compared to rest of the selected compounds and the standard. These results suggested that the F1 to F5 compounds possess the potential angiotensin converting enzyme inhibitory activity than the other selected flavonoids and the order of potency is found to be F1>F2>F3>F4>F5.

The other two parameters like inhibition constant (Ki) and intermolecular energy were also determined. Inhibition constant is directly proportional to binding

energy. The selected flavonoids showed inhibition constant ranging between-8.36 to -8.12 Nm. F1and F2 flavonoids showed excellent inhibition constant -8.36 and -8.15, standard enalapril 6.13 nM. F1 flavonoid showed higher inhibition constant when compared to other selected flavonoids. Intermolecular energy is also directly proportional to binding energy. Flavonoids showed intermolecular energy ranging between -6.23 to -7.13 kcal/ mol. F1and F2 flavonoid showed excellent intermolecular energy -6.23 and -7.13 standard enalapril -7.58 kcal/ mol.

Table7: *In silico* docking results of ACE [PDB ID:1086] inhibition by selected compounds

SL.NO	FLAVONOIDS	BINDING ENERGY	INHIBITION CONSTANT (nM)	INTERMOLECULAR ENERGY
1	F1	-8.36	255.13	-6.23
2	F2	-8.15	151.23	-7.13
3	F3	-6.09	259.3	-7.26
4	F4	-7.32	158.25	-7.23
5	F5	-8.12	360.28	-8.9
6	F6	-7.36	256.42	-7.26
7	F7	-6.90	465.23	-10.62
8	F8	-8.51	580.05	-11.19
9	F9	-7.68	2.35	-9.17
10	F10	-8.56	534.69	-9.45
11	F11	-9.4	128.03	-10.3
12	F12	-7.33	4.25	-8.22
13	F13	-7.49	198.05	-8.15
14	F14	-8.73	397.03	-10.22
15	F15	-8.92	288.21	-11.61
16	F16	-7.8	1.92	-9.29
17	F17	-8.48	604.32	-9.98
18	F18	-10.34	26.42	-12.13
19	F19	-8.12	1.12	-9.91
20	F20	-8.1	1.15	-9.89
21	F21	-7.69	2.31	-9.48

22	F22	-9.12	207.28	-10.91
23	F23	-9.29	153.75	-11.08
24	F24	-9.56	99.06	-11.94
25	F25	-10.05	42.66	-12.44
26	F26	-9.36	138.22	-10.55
27	F27	-9.07	224.37	-10.26
28	F28	-8.42	670.04	-10.21
29	F29	-8.47	616.62	-9.96
30	F30	-8.56	533.89	-10.05
31	F31	-8.16	1.04	-9.66
32	F32	-9.12	207.94	-10.91
33	F33	-8.6	498.09	-9.79
34	F34	-8.69	425.16	-11.08
35	F35	-8.85	328.39	-9.74
36	F36	-9.5	108.57	-10.4
37	F37	-7.45	3.46	-8.35
38	F38	-8.17	1.02	-10.56
39	F39	-7.49	3.24	-10.17
40	F40	-9.44	119.88	-10.64
41	F41	-10.44	22.07	-11.64
42	F42	-9.05	233.23	-10.24
43	F43	-10.15	36.48	-11.34
44	F44	-7.63	2.57	-10.01
45	F45	-8.24	917.79	-10.62
46	F46	-10.0	46.38	-11.5
47	F47	-7.9	1.61	-8.5
48	F48	-8.1	1.15	-8.7
49	F49	-7.04	6.93	-7.64
50	F50	-7.94	1.51	-8.84

F1-F5 compounds showed excellent inhibition constant and intermolecular energy when compared to rest of the selected compounds and the standard. These results suggested that the F1 to F5 compounds possess the potential angiotensin converting enzyme inhibitory activity than the other selected flavonoids and the order

of potency is found to be F1>F2>F3>F4>F5. These results further proved that the F1 to F5 compounds possess the better angiotensin converting enzyme inhibitory activity than the other selected flavonoids.

DOCKING ORIENTATIONS

Binding of selected flavonoids with ACE [1086]

The potential binding sites of the F1 was found that TYR334, PHE330, PHE331, PHE288,TRP279, SER288, LE287 and the binding sites of the F2 was found to be ASP72, TYR121, PHE288, ARG289, PHE330, PHE331

Figure 7: F1 – 2 Chloro & F2 – 4 Chloro

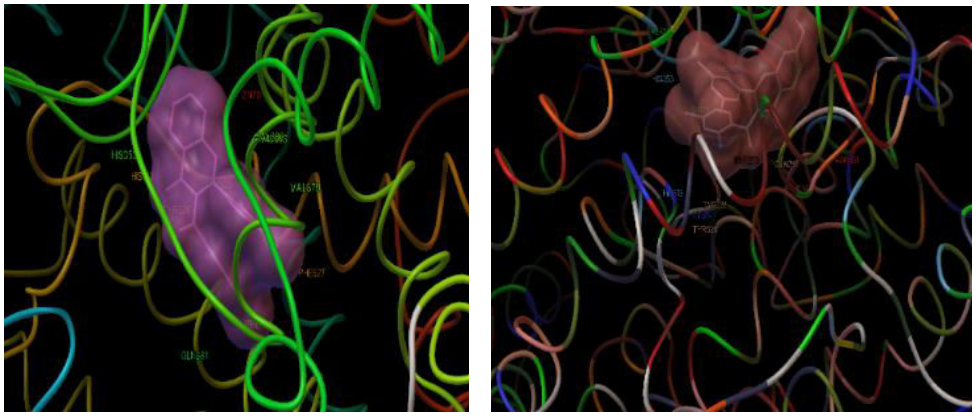


Figure 8: F3 – 2 ethoxy & F4 – 2,4 Dichloro

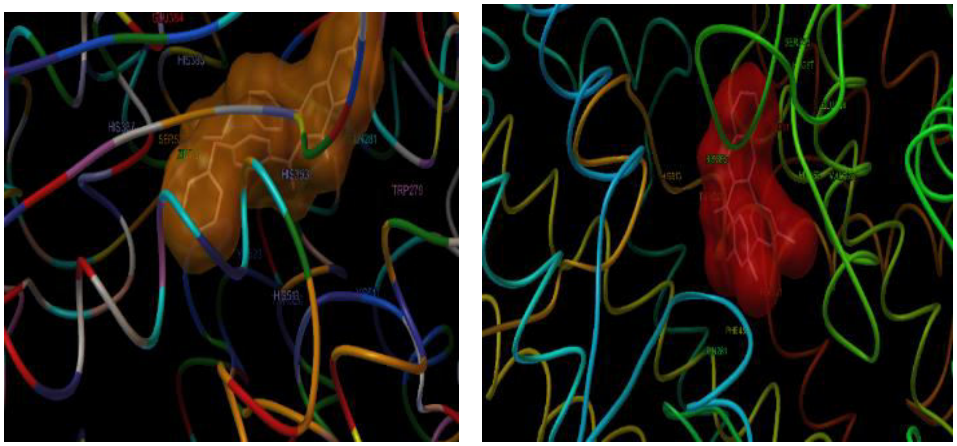
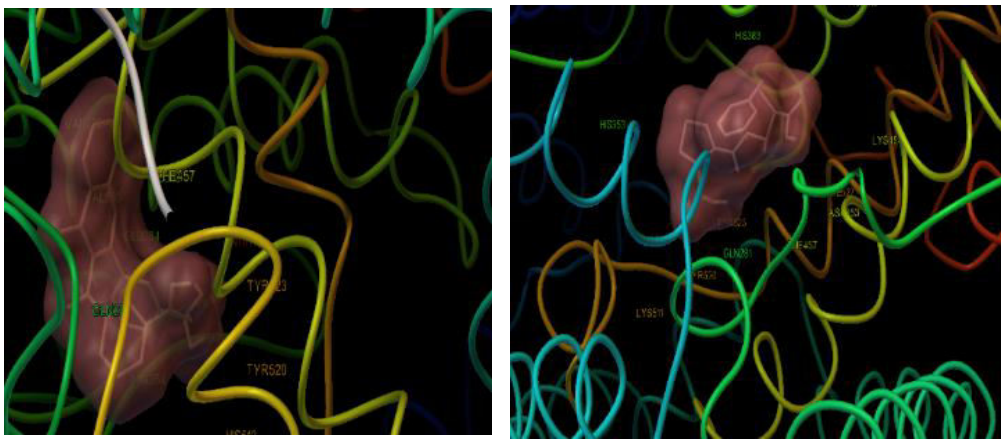


Figure 9: F5 – 2 Chloro, 5 Nitro & Enalapril



This proves that the effective binding sites were present in the selected flavonoids such as F1(2-ethoxy), F2 (2,4-dichloro), F3 (2-chloro), F4 (4-chloro), F5 (2-chloro, 5-nitro) when compared with the standard and also it shows the inhibitory potential of the angiotensin converting enzyme.

Table8: Docking orientations of the selected flavonoids and enalapril

C. code	Binding interaction with amino acid residue
F1	ZN707, HIS353, TYR520, TYR523, VAL360, VAL379, PHE527, GLN281
F2	ALA301, HIS353, HIS383, HIS513, ZN701, TYR523, TRP270, ASP423, GAN 281
F3	GLU384, HIS333, HIS387, HIS353, SER528, ZN701, TYR523, HIS513, LYS511, TRP279, GLN281
F4	SER385, HIS387, GLU224, HIS513, HIS353, PHE451, GLN281, PHE527, VAL260, ZN201
F5	VAL370, VAL330, TYR123, TYR520, GLN280, GLU384, ALA354
Enalapril	HIS383, HIS353, LYS511, TYR520, GLN281, TYR623, LYS450

The standard enalapril and selected flavonoids possess binding similarity of the aminoacids with the target. Due to this binding site simiarity, these flavonoids possess inhibitory activity towards angiotensin converting enzyme similar to that of standard. Since, the potential binding parameters were identified in the selected flavonoids in the *in silico* studies. So, the flavonoids has the chance to bind the amino acid residues in the target so effectively than the standard. This result further strenghthen the inhibitory potential of flavonoids than that of the standard. On the basis of the above study, all the selected flavonoids showed better angiotensin converting inhibitory activity. Among the selected flavonoids F1, F2, F3, F4 and F5 showed excellent binding interactions and orientations towards the target enzyme. This may be attributed due to the differences in the functional groups in these compounds.Thus, these compounds further selected for synthesis, *in vitro* and *in vivo* angiotensin converting enzyme inhibitory activity.

ADMET studies:

ADMET studies were carried out using MedChem Designer software for the compounds selected for synthesis and the results are tabulated in Table 9.

Table 9: ADMET properties of the synthesized compounds

C. CODE	FPSA	AQ.SO L.LOG	AQ. SOL. LOGLEV	BBB. DIST. T2	BBB LOG	BBB LOG.LEV	CYP2D6	CYP2D6. PROB	FST. ALOGP98	HEPATOTOX	HEPATOTOX.PROB	HIA. FABS.LEV	HIA. FABS.LEV T2	PROT. BIND.LEV	PROT. BIND.LEV.LOG
F1	45.3694	-0.235	2	0.30069	1.958	5	1	0.6823	2.056	1	0.94702	0	0.30816	2	0
F2	49.3657	2.615	3	0.19841	3.502	1	1	0.2586	2.581	1	0.953642	0	0.15842	2	0
F3	53.1285	3.198	3	6.12380	3.976	4	1	0.8535	4.430	1	0.980132	0	0.20103	2	0
F4	62.1895	3.411	2	0.22534	4.728	4	1	0.71287	2.985	1	0.953642	0	0.22181	2	0
F5	61.701	2.362	4	0.690774	3.425	3	1	0.5861	3.425	1	0.96026	0	0.00326	2	0

SYNTHESIS OF FLAVONOIDS

The percentage yield of all the synthesized compounds ranged from 75 to 86%. The melting point was determined by using MR-VIS visual melting range apparatus and the melting point ranged from 113.5°C to 235.5°C. The percentage yield, melting point of the synthesized flavonoids were provided.

Table10: Percentage yield and melting point and of synthesized flavonoids

Compound code	Percentage yield	Melting point
F1	75%	113.5°C
F2	76%	235.5°C
F3	87%	168.4°C
F4	75%	183.2°C
F5	86%	197.3°C

IN VITRO ACETYLCHOLINESTERASE INHIBITORY ASSAY

A total of 50 compounds were used for docking studies and based on the results of binding energies, inhibition constant and intermolecular energies, 5 compounds were selected and synthesized. The 5 synthesized flavonoids were tested for inhibition of angiotensin converting enzyme. The synthesized flavonoids were dissolved in water and suitable concentration (0.2 - 3.2µg/ml) was prepared based on the pilot study. Enalapril was used as reference standard for comparison of results of the test compounds. The absorbance of all the synthesized flavonoids (F1 to F5 and enalapril) decreased upon increasing the concentrations in a dose dependent manner. The percentage inhibition was calculated for all the synthesized flavonoids (F1 to F5) and for enalapril. A dose dependent increase in percentage of inhibition was observed for standard and synthetic flavonoids. The percentage inhibition of enalapril ranged between 25.62 to 100.13% and the IC_{50} was found to be 19.85 ± 25µg/ml. The percentage of inhibition for compound F1 (2-chloro) ranged from 49.55 to 101.08% and its $IC_{50}$was found to be 0.287 ± 0.13 µg/ml. For compound F2 (4-chloro) the percentage inhibition ranged from 32.27to 63.54% and the IC_{50} value was found to be 1.464 ± 0.23 µg/ml. The percentage inhibition of acetylcholinesterase enzyme with F3 (2-ethoxy) compound was found to be from 42.41 to 60.54 % and the IC_{50} was 0.803 ± 0.03 µg/ml. The F4 (2,4-dichloro) compound had IC_{50} value of 1.500 ± 0.16 µg/ml and its percentage inhibition ranged from 31.06 to 62.71. The percentage inhibition for F5 (2-chloro,5-nitro) ranged between 32.46 to 92.26% respectively and IC_{50} was found to be 0.618 ± 0.41µg/ml. Among the 5 synthetic compounds, F1 and F2 had greater percentage of inhibition at 3.2 µg/ml concentration with 101.08% activity. Excellent inhibitory activity was possessed by since for this compound the IC_{50} was 0.287 ± 0.13. The order of potency based on IC_{50} for synthesized flavonoids to angiotensin converting enzyme inhibitory activity was found to be F1>F2>F3>>F4>F5 respectively.

Table 11: Percentage inhibition of synthesized flavonoids to angiotensin converting enzyme

Concentration (µg/ml)	Percentage inhibition (%) of ACE					IC_{50}value
	F1	F2	F3	F4	F5	
50	27.94 ± 19	27.19 ± 23	56.58 ± 35	22.38 ± 22	56.52 ± 37	259.65 ± 16
100	40.07 ± 31	35.16 ± 37	57.41 ± 47	29.38 ± 37	59.34 ± 37	325.36 ± 17
200	48.15 ± 65	43.13 ± 35	76.03 ± 09	57.13 ± 36	73.61 ± 29	70.21 ± 35
400	52.59 ± 52	54.76 ± 09	80.60 ± 57	60.49 ± 21	80.08 ± 36	165.52 ± 22
800	73.89 ± 24	75.99 ± 57	88.05 ± 05	78.85 ± 18	90.58 ± 28	73.89 ± 24

All the determinations were carried out in triplicate and the values are expressed as the Mean ± SEM. F1, F2, F3, F4, F5 are different flavonoids. The standard drug enalapril.

SPECTRAL MEASUREMENTS OF SYNTHESIZED FLAVONOIDS

Compound code: F3

IUPAC NAME	3-(2-chloro benzoyl)-2-(2-chloro phenyl)-4H-chromen-4-one
UV- SPECTRUM	λ_{max} – 375nm (solvent – dimethyl sulphoxide)
IR SPECTRUM (KBr Cm^{-1})	3447.13(Ar C-H), 1675.84(C=O), 1474.31(Ar C=C),921.80 (C-O), 772.35(C-Cl)
MASS SPECTRUM	395.21 M^+ ion peak

Compound code: F4

IUPAC NAME	3-(4-chloro benzoyl)-2-(4-chloro phenyl)-4H-chromen-4-one
UV- SPECTRUM	λ_{max} –360 nm (solvent – dimethyl sulphoxide)
IR SPECTRUM (KBr Cm^{-1})	3393.13(Ar C-H), 1630.23(C=O),1424.02(Ar C=C), 943.60 (C-O), 738.53 (C-Cl)
MASS SPECTRUM	395.37, M^+ ion peak

IN VIVO STUDIES

Acute toxicity studies and selection of dose for *in vivo* studies

Among the 5 compounds selected for *in vitro* angiotensin converting enzyme inhibitory assay F3 and F4 flavonoids were found to possess good activity in inhibiting the enzyme and these compounds were selected and used for further *in vivo* assays. Acute toxicity was determined as per OECD guidelines (420) employing fixed dose procedure for selecting the dose for biological activity. For acute toxicity studies female *Wistar* rats weighing 180-200g were taken and they were fasted overnight before the experimental day. Overnight fasted rats were weighed and body weight determined for dose calculation and the test flavonoids (F3-2-chloro and F4-4-chloro) were administered orally. Sighting study was conducted with a lower dose of 5 mg/kg using 0.5% CMC. After administration, the animals were observed for occurrence of toxic effects. No toxic effect were observed and after sufficient interval of time (2-3 days) the second animals were administered with 50 mg/kg dose of test flavonoids. Similar observations were made as before and since the doses was non-toxic the third animal was administered with 300 mg/kg. This dose also did not exhibit any toxicity or morbidity and therefore the last and highest dose in fixed dose procedure of 2000 mg/kg were administered to fourth animal. No toxicity was noticed for highest doses. Signs and symptoms of toxicity and death if any were observed individually for each rat at 0, 0.5, 1, 2, 3 and 4 h for first 24 h and thereafter daily for 14 days. Diet was given to the animals after the 4^{th} hours of dosing. The animals were observed twice daily for 14 days and body weight changes, food and water consumption etc., were noted. In acute toxicity studies, it was found that the animals were safe up to a maximum dose of 2000 mg/kg of body weight. There were no changes in normal behavioural pattern and no signs and symptoms of toxicity and mortality were observed. As per the OECD 420 guidelines F3-2-chloro flavonoid and F4-4-chloro can be included in the category 5 or unclassified category of globally harmonized classification system (GHS). Hence, based on these results the F3 (2-chloro) and F4(4-chloro) flavonoids were considered non-toxic and $1/10^{th}$ and $1/20^{th}$ dose were used for the biological evaluation (cardioprotective activity) and the studies were conducted at dose levels of 100 and 200 mg/kg body weight.

Table 12: OBSERVATIONS DONE FOR THE ACUTE ORALTOXICITY STUDY- F3 (2-CHLORO) & F4 (4-CHLORO) COMPOUNDS

Observation	Score for Normal animal	Half an hour	Four hours	24 Hours	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Lethality		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Group Dispersion	0	0	0	0	0	0	0	0	0	0	0
Locomotor Activity	4	4	4	4	4	4	4	4	4	4	4
Restlessness	0	0	0	0	0	0	0	0	0	0	0
Lethargy	0	0	0	0	0	0	0	0	0	0	0
Writhing	0	0	0	0	0	0	0	0	0	0	0
Stereotyped behavior	0	0	0	0	0	0	0	0	0	0	0
Tremors	0	0	0	0	0	0	0	0	0	0	0
Twitches	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0
Exophthalmos	0	0	0	0	0	0	0	0	0	0	0
Respiration	0	0	0	0	0	0	0	0	0	0	0
Alertness	4	4	4	4	4	4	4	4	4	4	4
Group startle response	4	4	4	4	4	4	4	4	4	4	4
NAD		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Observation	Score for normal animal	Time of individual observations									
		Half an hour	Four hours	24 Hours	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Loss if Righting reflex	0	0	0	0	0	0	0	0	0	0	0
Abnormal body carriage	0	0	0	0	0	0	0	0	0	0	0
Abnormal gait	0	0	0	0	0	0	0	0	0	0	0
Straub tail	0	0	0	0	0	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0
Touch response	4	4	4	4	4	4	4	4	4	4	4
Fearfulness	4	4	4	4	4	4	4	4	4	4	4
Pinna reflex	4	4	4	4	4	4	4	4	4	4	4
Corneal reflex	4	4	4	4	4	4	4	4	4	4	4
Catalepsy	0	0	0	0	0	0	0	0	0	0	0
Passivity	0	0	0	0	0	0	0	0	0	0	0
Aggressiveness	0	0	0	0	0	0	0	0	0	0	0
Body tone	4	4	4	4	4	4	4	4	4	4	4
Grip strength	4	4	4	4	4	4	4	4	4	4	4
Paralysis	0	0	0	0	0	0	0	0	0	0	0
Cutaneous blood flow	4	4	4	4	4	4	4	4	4	4	4
Cyanosis	0	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	0	0	0
Lacrimation	0	0	0	0	0	0	0	0	0	0	0
Ptosis	0	0	0	0	0	0	0	0	0	0	0
Pupil diameter	4	4	4	4	4	4	4	4	4	4	4
Pain response	4	4	4	4	4	4	4	4	4	4	4
Diarrhea	0	0	0	0	0	0	0	0	0	0	0
Vocalization	0	0	0	0	0	0	0	0	0	0	0
Increased urination	0	0	0	0	0	0	0	0	0	0	0
Grooming	4	4	4	4	4	4	4	4	4	4	4
NAD		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Table13: MORTALITY RECORD FOR F3(2-CHLORO) COMPOUND IN ACUTE ORAL TOXICITY STUDY

Dose	Sighting study				Main study				
	5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg
No. of animals	1	2	3	4	5	6	7	8	9
Body weight (g)	188	188	184	196	189	190	193	192	188
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female
30 min	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
1 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
2 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
3 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
4 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 1	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 2	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 3	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 4	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 6	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 7	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 8	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 9	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

continued

Dose	Sighting study				Main study				
	5 mg/kg	**50 mg/kg**	**300 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**
No. of animals	1	2	3	4	5	6	7	8	9
Body weight (g)	189	188	187	190	195	190	193	192	190
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female
Day 10	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 11	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 12	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 13	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 14	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Mortality	0/0	0/0	0/0	0/0	0/0				

Table14: MORTALITY RECORD FOR F4 (4-CHLORO) COMPOUND IN ACUTE ORAL TOXICITY STUDY

Dose	Sighting study				Main study				
	5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg	2000 mg/kg
No. of animals	1	2	3	4	5	6	7	8	9
Body weight (g)	188	179	188	196	199	190	189	189	188
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female
30 min	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
1 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
2 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
3 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
4 h	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 1	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 2	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 3	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 4	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 6	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 7	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 8	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 9	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Dose	Sighting study				Main study				
	5 mg/kg	**50 mg/kg**	**300 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**	**2000 mg/kg**
No. of animals	1	2	3	4	5	6	7	8	9
Body weight (g)	193 176	178 176	186 187	192 195	200 198	190 187	190 193	192 194	190 191
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female
Day 10	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 11	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 12	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 13	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Day 14	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Mortality	0/0	0/0	0/0	0/0	0/0				

Table 15: Effect of F3 (2-chloro) and F4 (4-chloro) flavonoids on body weight in doxorubicin-induced myocardial infarction in rats

Dose	Body weight (g)	
	Initial	Final
0.5%w/v of CMC	147.83±5.16	167.16±5.55^{ns}
Doxorubicin (10mg/g)	187.66±9.26	197.66±5.17*
F3-2-chloro (100mg/k)	178.16±4.91	184.10±5.17^{ns}
F3-2-chloro (200mg/kg	186.10±5.22	195.33±3.13^{ns}
F4-4-chloro (100mg/kg	174.16±3.92	189.16±6.50^{ns}
F4-4-chloro (200mg/kg)	174.33±4.44	195.10±6.69^{ns}
Enalapril (10mg/kg)	176.33±3.82	198.16±4.10^{ns}

Values are expressed as mean ± SEM (n=6).

Values in parentheses are the percent increase or decrease from their corresponding initial readings.

* denotes $p < 0.05$; ^{ns} denotes $p > 0.05$ when compared to initial readings; when compared to the initial readings (Student's Paired't'-test);

Table 16: EFFECT OF 2-CHLORO AND 4-CHLORO FLAVONOIDS ON SERUM MARKERS IN DOXORUBICIN-INDUCED MYOCARDIAL INFARCTION IN RATS

DOSE	MARKERS OF MI			LIVER FUNCTION TESTS		
	CK-MB (IU/L)	LDH (IU/L)	TROPONIN- I	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
0.5%w/v of CMC	1786.25 ± 232.54	55.85 ± 9.013	-	7.50 ± 6.51	35.29 ± 32.67	595.38 ± 110.81
Doxorubicin (10mg/g)	3259.30 ± 412.62$^{\#}$	71.39 ± 4.907$^{\#}$	+	249.66 ± 4.30**	505.05 ± 0.92$^{\#\#\#}$	1720.41 ± 119.25$^{\#\#}$
F3-2-chloro (100mg/k)	1918.17 ± 372.46*	37.37 ± 2.053*	-	41.19 ± 3.80***	4.38 ± 1.08***	990.40 ± 126.57*
F3-2-chloro (200mg/kg	1748.16 ± 215.24*	28.37 ± 1.747**	-	32.92 ± 6.04***	6.94 ± 2.79***	385.06 ± 95.98***
F4-4-chloro (100mg/kg	2875.13 ± 461.90*	33.65 ± 0.919*	-	49.41 ± 4.24***	8.28 ± 1.34***	610.48 ± 81.16**
F4-4-chloro (200mg/kg)	2576.26 ± 371.45*	26.33 ± 3.088**	-	2.20 ± 4.24***	37.39 ± 31.97***	787.71 ± 41.02**
Enalapril (10mg/kg)	2907.60 ± 334.29**	47.51 ± 3.71**	-	2.78 ± 0.26**	17.3 ± 4.61**	587.23 ± 17.87**

Values are expressed in mean ± SEM (n=6)

# denotes $p < 0.05$, ## denotes $p < 0.01$ and ### denotes $p < 0.001$ when compared to vehicle control;

*denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control (one way ANOVA followed by Tukey's test0)

Table 17: EFFECT OF 2-CHLORO AND 4-CHLORO FLAVONOIDS ON LIPID PROFILE IN DOXORUBICIN-INDUCED MYOCARDIAL INFARCTION IN RATS

DOSE (mg/kg)	TRIGLYCERIDES	HDL	LDL	VLDL
0.5%w/v of CMC	193.29 ± 17.11	22.33 ± 0.52	68.2 ± 0.46	38.65 ± 3.42
Doxorubicin 10mg/kg	973.68 ± 17.39$^{\#\#}$	6.12 ± 1.17$^{\#\#}$	98.60 10.62$^{\#\#}$	174.73 ± 3.47$^{\#}$
F3-2-chloro (100mg/kg)	153.40 ± 11.28**	50.07 ± 2.48***	61.31 9.11**	30.68 ± 2.25***
F3-2-chloro (200mg/kg	152.35 ± 18.35**	17.27 ± 1.813***	69.23 ± 6.11**	30.46 ± 3.67***
F4-4-chloro (100mg/kg	184.24 ± 17.45*	21.68 ± 0.94***	51.33 ± 5.20**	36.84 ± 3.49***
F4-4-chloro (200mg/kg	147.53 ± 18.93**	35.30 ± 1.55**	60.25 ± 20**	29.50 ± 3.78***
Enalapril (10mg/kg)	212.99 ± 12.98**	14.81 ± 2.89**	71.53 3.20**	42.59 ± 2.59**

Values are expressed in mean ± SEM (n=6)

# denotes $p < 0.05$ and ## denotes $p < 0.01$ when compared to vehicle control;

*denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control (One way ANOVA followed by Tukey's test)

Table 18: EFFECT OF F1 (2-CHLORO) AND F4 (4-CHLORO) FLAVONOIDS ON TOTAL PROTEIN & MDA IN HEART TISSUE OF DOXORUBICIN-INDUCED MYOCARDIAL INFARCTION IN RATS

DOSE	TOTAL PROTEN (mmoles/min/mg)	MDA (µmoles/min/mg)
0.5%w/v of CMC	2.20 ± 0.10	17.46 ± 0.60
Doxorubicin 10mg/kg	0.85 ± 0.11$^{\#}$	48.17 ± 0.74$^{\#\#}$
2-chloro (100mg/kg)	1.30 ± 0.10*	15.25 ± 0.52**
2-chloro (200mg/kg	1.50 ± 0.12*	14.36 ± 0.62**
4-chloro (100mg/kg	1.08 ± 0.12*	11.52 ± 0.53**
4-chloro (100mg/kg	1.80 ± 0.18*	12.367 ± 0.62**
Enalapril (10mg/kg)	2.03 ± 0.15*	15.25 ± 0.25**

Values are expressed in mean ± SEM (n=6).

$^{\#}$ denotes $p < 0.05$ and $^{\#\#}$ denotes $p < 0.01$ when compared to vehicle control; * denotes $p < 0.05$ and ** denotes $p < 0.05$ when compared to negative control. (One way ANOVA followed by Tukey's test).

Table 19: EFFECT OF 2-CHLORO AND 4-CHLORO FLAVONOIDS ON ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS IN THE HEART TISSUE OF DOXORUBICIN-INDUCED MYOCARDIAL INFARCTION IN RATS

DOSE (mg/kg)	ENZYMATIC ANTIOXIDANTS				NON-ENZYMATIC ANTIOXIDANT
	SOD (µmoles/min/mg protein)	CAT (mmoles/min/mg protein)	GPX (µmoles/min/mg protein)	GSSH (µmoles/min/mg protein)	GSH (µmoles/min/mg protein)
0.5%w/v of CMC	36.71 ± 0.807	60.568 ± 2.12	14.365 ± 0.44	15.89 ± 0.44	14.54 ± 0.25
Doxorubicin 10mg/kg	2.390 ± 1.31$^{\#\#}$	4.064 ± 2.02$^{\#\#}$	1.83 ± 0.13$^{\#\#}$	1.40 ± 0.0036$^{\#\#}$	1.52 ± 0.08$^{\#\#}$
F3-2-chloro (100mg/kg)	25.060 ± 0.10*	55.358 ± 0.17**	13.120 ± 0.12**	11.78 ± 0.39***	13.550 ± 0.14**
F3-2-chloro (200mg/kg	44.957 ± 0.09**	56.808 ± 0.27**	13.768 ± 0.22**	11.84 ± 0.33***	14.680 ± 0.19**
F4-4-chloro (100mg/kg	36.030 ± 0.20*	59.96 ± 0.13**	15.123 ± 0.21**	8.268 ± 0.12**	14.251 ± 0.023**
F4-4-chloro (200mg/kg	55.065 ± 0.05***	61.89 ± 0.23***	15.656 ± 0.32**	11.177 ± 0.05***	15.142 ± 0.025**
Enalapril (10mg/kg)	57.135 ± 0.11***	63.905 ± 0.24***	13.505 ± 0.28**	10.864 ± 1.35***	14.673 ± 0.16**

Values are expressed in mean ± SEM (n=6)

# denotes $p < 0.05$ and ## denotes $p < 0.01$ when compared to vehicle control;*denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control (One way ANOVA followed by Tukey's test).

Effect of synthesized flavonoids on cardiac markers and liver function tests

CK-MB, LDH and troponin-I levels were measured in all treatment groups. In DOX alone (negative control) treated rats a significant elevation (3259.30 ± 412.62) of markers were observed confirming the occurrence of MI on comparison to control group. Treatment with F3-2-chloro-100 and F3-2-chloro-200 mg/kg doses resulted in reduction of cardiac markers. The level of CK-MB in F3-2-chloro and F4-4-chloro was found to be 1918.17 ± 372.46 and 1748.16 ± 215.24 at 100 and 200 mg/kg dose. Similarly, with F4 (100 mg/kg), the level of CK-MB at 100 mg/kg dose was found to be 2875.13 ± 461.90 and at 200 mg/kg dose (F4) it decreased to 2576.26 ± 371.45. The level of CK-MB for standard drug (enalapril) treatment was found to be 2907.60 ± 334.29. LDH levels were also decreased in F3 (100 mg/kg) to 37.37 ± 2.05 and in F3 (200 mg/kg) it further reduced to 28.37 ± 1.74 and this shows higher concentration a better effect. Likewise, in F4 (100 mg/kg) and F4 (200 mg/kg) treated group a significant reduction of LDH was noticed with values of 33.65 ± 0.919 and 26.33 ± 3.08 respectively. The compounds F4 (100 mg/kg) and F4 (200 mg/kg) reduced LDH levels effectively compared to F3 (100 mg/kg) and F3 (200 mg/kg). The troponin-I levels were decreased in all treatment groups when compared to negative control group showing the occurrence of MI. The level of ALT, AST and ALP is increased during MI and these levels were effectively reduced on treatment with synthesized compounds F3 (100 mg/kg), F3 (200 mg/kg), F4 (100 mg/kg) and F4 (200 mg/kg).

Estimation of serum lipid profile in rats treated with flavonoids

MI infarction induction with DOX resulted in elevation of triglycerides, total cholesterol, LDL and VLDL and reduction of HDL levels. Triglycerides level in negative control group was found to be 193.29mg/dl when compared to control 173.68 mg/dl. Treatment with F3 at both doses reduced the triglyceride values significantly to 153.40 and 152.35 ± 06 mg/dl. The results obtained with F4 (100 mg/kg) and F4 (200 mg/kg) was found to be 184.24 and 147.53 mg/dl and it can be confirmed that F4 at 200 mg/kg dose level effectively reduced the triglyceride level. The level of bad cholesterol (LDL) was found to be higher for negative control group 98.60 mg/dl and treatment with F4 (100 mg/kg) and F4 (200 mg/kg) markedly lowered the levels compared to negative control group with values of 51.33 and 60. 25 mg/dl when compared to F3 (100 mg/kg) and F3 (200 mg/kg) with 61.31 and 69.23 respectively.

Better control was noticed for F4 (100 mg/kg) and F4 (200 mg/kg). The level of HDL was reduced in negative control group and treatment with synthesized flavonoids elevated the levels. Elevation of HDL was noticed in F4 (200 mg/kg) group to 21.68 and 17.27 in F4 (100 mg/kg) and group and effective increase in the levels were noticed compared to negative control treated rats.

Estimation of MDA levels in heart tissue homogenate

The end product of lipid peroxidation, MDA was elevated in negative control group to 48.173 and treatment with F3 (100 mg/kg) the MDA level reduced to 15.25 and with F3 (200 mg/kg) it was found to be 14.36. Reduction in MDA level was observed for F4 (100 mg/kg) and F4 (200 mg/kg) and the values were found to be 12.36 and 11.52. Treatment with synthesized flavonoids for 28 days effectively reduced the MDA level and this marks that the test compounds has the potential to control lipid peroxidation during occurrence of MI with DOX.

Antioxidant status of synthesized flavonoids

The enzymatic antioxidants SOD, CAT, GPX and GSSH levels were reduced in negative control group. This denotes that MI occurrence after DOX administration. Treatment with synthesized flavonoids markedly elevated the levels of enzymatic antioxidants such as SOD, CAT, GPX and GSSH levels and occurrence of MI is markedly reduced in treatment groups and this is noticed by an increase in levels of all enzymatic antioxidants in heart tissue homogenates. The non-enzymatic antioxidant such as GSH was found to be reduced in negative control group when compared to normal control. Treatment with F3 (100 mg/kg), F3 (200 mg/kg), F4 (100 mg/kg) and F4 (200 mg/kg) significantly restored the elevated level of GSH. The treatment groups were compared with standard drug enalapril and a similar increase in levels of antioxidant levels were noticed.

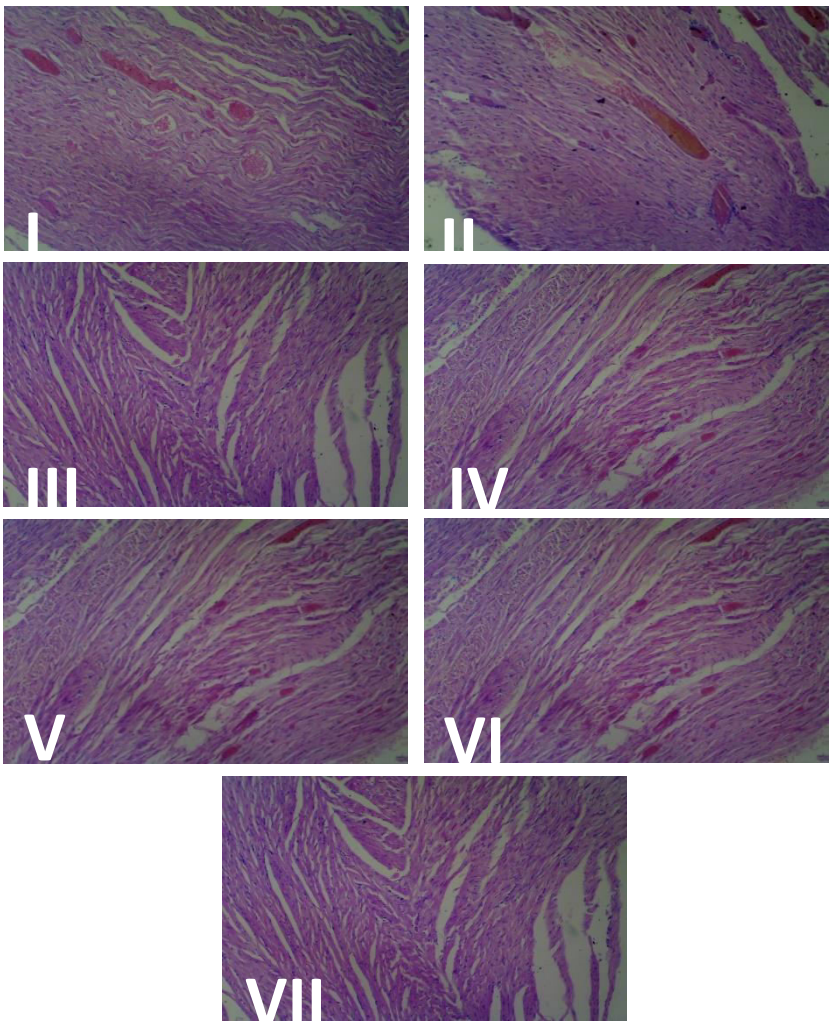


Figure 10: Histopathology of heart tissue

Group I (control) rats -normal myocardium with normal myocytes and no evidence of architectural destruction/ oedema/ inflammatory infiltrates/ necrosis.

Group II **(**Negative control) severe architectural destruction, edematous stroma, scattered infiltration of lymphocytes and extravasated RBC and areas of haemorrhage with congested blood vessels.

Group III – F3 (100 mg/kg) myocardium shows mild loss of striation and stroma with pigmented laden macrophages, lymphocytic infiltration and haemorrhage with congested blood vessels.

Group IV – F3 (200 mg/kg) shows normal architecture of myocardium with infiltration of stroma.

Group V- F4 (100 mg/kg) exhibits normal architecture of myocardium with scattered lymphocytic infiltrations and the mild congestion of blood vessels.

Group VI- F4 (200 mg/kg) showed heart with normal regular architecture and normal myocytes. There was no evidence of architectural destruction/ necrosis.

Group VII – standard drug- normal myocardium architecture with normal myocyte and no evidence of architectural destruction/ oedema/ inflammatory infiltrates/ necrosis.

Group I (control) rats has shown heart with normal myocardium with normal myocytes. The blood vessels showed mild congestion and there was no evidence of architectural destruction/ oedema/ inflammatory infiltrates/ necrosis.

Group II (Negative control) has shown myocardium with severe architectural destruction. Stroma was edematous with scattered infiltration of lymphocytes and extravasated RBC. The blood vessels showed significant pathology.

The heart sections of **Group III** - FI (100 mg/kg) showed myocardium with mild loss of striation and stroma showed pigmented laden macrophages, lymphocytic infiltration and areas of hemorrhage with congested blood vessels.

Group IV - FI (200 mg/kg) treated rats has shown myocardium with normal architecture and infiltration of stroma was noticed and the blood vessels exhibited no significant pathology.

Group V- F2 (100 mg/kg) heart exhibited myocardium with normal architecture. Stroma was scattered with lymphocytic infiltrations and the blood vessels showed mild congestion.

Group VI- F2 (200 mg/kg) the sections showed heart with normal myocardium with normal regular architecture and normal myocytes. The blood vessels showed no congestion. There was no evidence of architectural destruction/ necrosis.

Group VII – standard drug treated rats shows heart with normal myocardium with normal regular architecture with normal myocyte. The blood vessels were normal and there was no evidence of architectural destruction/ edema/ inflammatory infiltrates/ necrosis.

DISCUSSION

BIOCHEMICAL ESTIMATION OF SERUM PARAMETERS

MARKERS FOR MYOCARDIAL INFARCTION

The present study revealed doxorubicin treated rats showed significant increase in the levels of diagnostic marker enzymes like CK-MB, LDH and troponin I in the serum and this may be due to the leakage of enzymes from the heart as a result of necrosis induced by doxorubicin[77]. The elevated levels of these enzymes are an indicator of the severity of doxorubicin-induced myocardial damage. The prior administration of F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg), showed significant reduction in doxorubicin -induced elevated CK-MB, LDH and troponin I serum marker enzymes. This reduction in the enzyme level confirms flavonoid I 2-chloro and flavonoids II 4-chloro (100, 200 mg/kg), that is responsible for maintenance of normal structural and architectural integrity of cardiac myocytes, thereby restricting the leakage of these enzymes, which can be accounted for membrane stabilizing property[48].

LIVER FUNCTION TESTS

This study shows the liver enzymes like AST, ALT and ALP enzyme activities are important measures of both early and late phases of cardiac injury. It is reported that serum AST, ALT and ALP were increased after Doxorubicin administration. ALP activity on endothelial cell surface is responsible, in part, for the conversion of adenosine nucleotides to adenosine, a potent vasodilator and anti-inflammatory mediator that can protect tissues from the ischemic damage that results from injury. This may account for the elevation of ALP in the Doxorubicin treated group, where tissue injury and inflammation are prominent. The evaluation of these enzymes together may be an indication of myocardial injury. In the preventive group, i.e. F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) and doxorubicin, the AST, ALT and ALP enzyme levels were decreased to a level near to that of control group, suggesting that F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) may protect the myocardial tissue against Dox toxicity[49].

BIOCHEMICAL ESTIMATION OF SERUM PARAMETERS

LIPID PROFILE

Lipid consist of triglycerides (neutral fat), total cholesterol, HDL and LDL and VLDL cholesterol. Cardiotoxicity is also related with the altered lipid metabolism. Increase in cholesterol level was observed due to the decrease in HDL, since HDL is known to be involved in the transport of cholesterol from tissues to the liver for its catabolism. The cholesterol-lowering drugs could significantly reduce the risk of heart attack by lowering the cholesterol level but they do not react with the free radical produced by doxorubicin, and hence they failed to exert protective action against doxorubicin-induced cardiotoxicity[87].

Increase in triglycerides might be due to a decrease in the activity of lipoprotein lipase, resulting in decreased uptake of triglycerides from the circulation and decreased level of phospholipid was observed due to enhanced membrane degradation in doxorubicin-treated rats. These changes in lipid levels might be due to enhanced lipid biosynthesis by cardiac cyclic adenosine monophosphate. Pre-treatment with F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) decreases the concentration of total cholesterol, TGs, VLDL and increases the concentration of HDL in the heart of doxorubicin-induced rats. Studies have shown that high levels of LDL cholesterol have a positive correlation with myocardial infarction, whereas high levels of HDL cholesterol have a negative correlation with myocardial infarction. The level of these enzymes is decreased in the serum of F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) pre-treated doxorubicin-induced animal due to the protective action of F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) which reduces the damage of myocardium and prevents the leakage of marker enzymes in the serum[87].

BIOCHEMICAL ESTIMATION OF HEART TISSUE PARAMETERS

TOTAL PTOTEIN & MDA

The mechanism of cardiotoxicity induced by doxorubicin is not clearly known from the present study, although large body of evidence indicates toward the formation of oxygen free radicals, which can damage cells by lipid peroxidation. In doxorubicin treated animals, we found significant increase in heart tissue malondialdehyde (MDA) levels suggesting increased lipid peroxidation. Cardiac tissue damage may be due to increased oxidative stress and depletion of antioxidants. In our study, doxorubicin

treated rats showed increase in heart tissue malondialdehyde (MDA) and total protein levels which confirms the oxidative stress and cardiac damage. F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) prevented the Dox-induced changes in malondialdehyde (MDA) and total protein suggests the protective effect of these flavonoids[78].

ENZYMATIC ANTIOXIDANTS

The doxorubicin treated rats showed decrease in levels the of enzymatic antioxidants like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), reduced glutathione (GSSH), and non-enzymatic antioxidant GSH which confirms the oxidative stress and cardiac damage. The treatment of F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) prevented the Dox-induced changes in MDA and enzyme levels. Significant increase in the GSH, SOD and CAT activity and decrease in lipid peroxidation in heart tissue of F3 (2-chloro) and F4 (4-chloro) (100, 200 mg/kg) and doxorubicin treated groups was found that, study suggests the protective effect of flavonoids. It is commonly accepted that SOD protects against the free radical injury by converting O_2- radical to H_2O_2 and prevent the formation of OH radicals through O_2- driven Fenton reaction and the H_2O_2 can be removed by catalase. Administration of these flavonoids improved the antioxidant status and thereby preventing the damage to the heart, mainly because of the antioxidant sparing action[78].

CONCLUSION

Flavonoids are polyphenolic compounds having certain biological activities such as anti-inflammatory, anticancer, anti-viral, anti-thrombotic, anti-ischaemic and also cardioprotective activity, because of its wide variety of pharmacological actions and it can be synthesized and their structural identification was done by UV, IR, and Mass spectroscopic techniques. Therefore, identification and characterization of flavonoid moieties is a promising approach against myocardial infarction.

Based *in silico* do0cking, molinspiration-drug likeness properties and ADMET checking five flavonoids were selected and synthesized. The angiotensin converting enzyme was isolated from the sheep lungs and the synthesized compounds were tested for *in vitro* inhibitory property of angiotensin converting enzyme. The *in vivo* cardioprotective activity were tested by inducing myocardial infarction using doxorubicin.

Based on the *in vitro* angiotensin converting enzyme inhibitory assay, it was found that two of the synthesized flavonoids (2-chloro and 4-chloro) possess cardioprotective activity. These 2 flavonoids showed maximum inhibition at lower concentration. *In vivo* studies were done for two flavonoids (2-chloro and 4- chloro) by doxorubicin induced myocardial infarction in rats. They possess a protection against doxorubicin induced myocardial infarction in rats. The present study suggests the protection offered by antioxidant enzymes and lipid peroxidation and reactive oxygen species and also, mitochondrial dysfunction.

The concluded results indicated that administration of doxorubicin leads to myocardial infarction moreover, from the biochemical and histopathological parameters, the present investigation suggest that 2-chloro and 4-chloro synthesized flavonoids can protect the doxorubicin induced myocardial infarction in rats. More advanced cohort studies are thus necessary to understand the absolute risk of doxorubicin and their mechanism for myocardial infarction and further extensive preclinical research of the synthesized flavonoids on the molecular and cellular targets have to be established before these chemical moieties are taken for clinical trials.

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